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Attorney for Plaintiff AntiCancer, Inc.

UNITED STATES DISTRICT COURT

FOR THE SOUTHERN DISTRICT OF CALIFORNIA

ANTICANCER, INC., a California corporation, Plaintiff, vs. PFIZER, INC., a Delaware corporation; CROWN BIOSCIENCE, INC., a California corporation, and DOES 1-10, Defendants.	Case No. 11 CV 0107 JLS (RBB) FIRST **AMENDED** COMPLAINT FOR: BREACH OF LICENSE AGREEMENT; BREACH OF IMPLIED COVENANT OF GOOD FAITH AND FAIR DEALING; UNJUST ENRICHMENT; PATENT INFRINGEMENT DEMAND FOR JURY TRIAL

AntiCancer, Inc., by and through its counsel, alleges for its First Amended Complaint against Pfizer Inc., Crown Bioscience, Inc., and Does 1-10, inclusive as follows:

JURISDICTION AND VENUE

1. This Court has jurisdiction over this action pursuant to 28 U.S.C. § § 1331, 1332(a)(1), 1338(a), and 2201.

2. Venue is proper in this judicial district under pertinent law, including, *inter alia*, 28 U.S.C. § 1391.

THE PARTIES

3. Plaintiff AntiCancer, Inc. ("AntiCancer") is a corporation organized and existing under the laws of the State of California and having as its principal place of business San Diego, California. AntiCancer has developed patented experimental mouse models and imaging techniques used by cancer researchers to test new anti-cancer drugs. AntiCancer licenses this (and other) technology to both large and small research organizations, including several major pharmaceutical companies.

4. Defendant Pfizer, Inc. ("Pfizer") is a corporation organized and existing under the laws of the State of Delaware and having as its principal place of business New York City, New York. In or around 2009, Pfizer wholly acquired Wyeth Pharmaceuticals ("Wyeth"), including Wyeth's Pharmaceuticals Division.

5. Defendant Crown Bioscience, Inc. ("Crown," also known as "CrownBio") is a corporation organized and existing under the laws of the State of California and having as its principal place of business Santa Clara, California. Crown provides research services to the pharmaceutical industry, including to Pfizer.

6. The true names and capacities, whether individual, corporate, associate, representative or otherwise, of DOES 1 through 10, inclusive, are unknown to plaintiff, who therefore sues them by such fictitious names. Plaintiff will seek leave to amend this complaint to show the true names and capacities of said defendants when they are ascertained. Plaintiff is informed and believes, and thereupon alleges, that each of the defendants named as a Doe, along with the named defendants, is responsible in some manner for the occurrences herein alleged, and that plaintiff's injuries herein alleged were legally or proximately caused by said defendants. Wherever it is alleged that any act or omission was also done or committed by any specifically named defendant, or by defendants generally, plaintiff intends thereby to allege, and does allege, that the same act or omission was also done and committed by each and every defendant named as a Doe, and each named defendant, both separately and in concert or conspiracy with the named defendants.

/ /

7. At all times mentioned herein, defendants, and each of them, were the agents, servants, co-conspirators, or employees of one another, and the acts and omissions herein alleged were done or suffered by them, acting individually and through or by their alleged capacity, within the scope of their authority. Each of the defendants aided and abetted and rendered substantial assistance in the accomplishment of the acts complained of herein. In taking the actions, as particularized herein, to aid and abet and substantially assist in the commission of the misconduct complained of, each defendant acted with an awareness of his, her or its primary wrongdoing and realized that his, her or its conduct would substantially assist in the accomplishment of that misconduct and was aware of his, her or its overall contribution to, and furtherance of the conspiracy, common enterprise, and common course of conduct. Defendants' acts of aiding and abetting included, *inter alia*, all of the acts each defendant is alleged to have committed in furtherance of the conspiracy, common enterprise, and common course of conduct complained of herein.

FACTS

8. On December 22, 2006, AntiCancer and Wyeth Pharmaceuticals entered into a technology licensing agreement ("License Agreement"). Certain portions of the License Agreement may contain confidential information. AntiCancer will seek leave of the Court to enter the License Agreement into the record under seal.

9. The License Agreement required AntiCancer to provide a limited, non-exclusive license to certain of its patented technologies, in exchange for Wyeth paying an initial fee of $250,000 and annual maintenance fees of $150,000 each. The annual maintenance fees are due on the December 22 anniversary of the License Agreement, and payable within fifteen (15) days of that date, i.e., January 6. Wyeth paid the initial fee, and for the next two years paid the required yearly maintenance fees.

10. The License Agreement also required AntiCancer to supply Wyeth with certain of its technical information ("Know-How"), cancer cell lines ("Cell Lines"), and mouse models ("Animal Models"). AntiCancer performed all of its requirements under the License Agreement.

11. In or around October 2009 Wyeth was acquired as a wholly-owned subsidiary of Pfizer. In December 2009, Pfizer affirmed its obligation to perform all of Wyeth's obligations under the License Agreement by paying the third annual maintenance fee.

12. On December 8, 2010, Beth E. Rosado, a Pfizer Operations Associate, sent an email to AntiCancer in which she stated "I am in the process of drafting a termination letter for a license agreement between Wyeth (now Pfizer, Inc.) and AntiCancer, Inc. dated December 22, 2006." (The December 8 email is attached hereto and incorporated herein as Exhibit 1).

13. On December 15, 2010, Ms. Rosado again emailed AntiCancer, this time attaching what she described as a "termination letter." (The December 15 email and its attached letter are attached hereto and incorporated herein as Exhibit 2).

14. The "termination letter" attached to Ms. Rosado's December 15 email was signed by Robert T. Abraham, Ph.D., Vice President and Chief Scientific Officer of Pfizer's Center for Integrative Biology and Biotherapeutics. Dr. Abraham's letter purported to be dated December 8, 2010. In it, Dr. Abraham stated that "Pursuant to Section 9.2 of the [December 22, 2006] Agreement, Pfizer hereby gives notice of termination of the Agreement. The Agreement shall therefore terminate on December 8, 2010." (Page 2 of Exhibit 2).

15. Section 9.2 of the License Agreement permits the Licensee to terminate the agreement "upon thirty (30) days prior written notice to Licensor." Pfizer's notice, whether deemed to be made on December 15 (the date it was received by AntiCancer) or December 8 (the date Pfizer purports the License Agreement to terminate), was given too late to relieve Pfizer of its obligation to pay the annual maintenance fee.

16. On information and belief, Pfizer knowingly and fraudulently backdated its termination letter in an attempt to induce AntiCancer to believe Pfizer had actually complied with the termination notice requirements. Pfizer did this for the sole purpose of unfairly withholding from AntiCancer the 2010 maintenance fee AntiCancer is entitled to under the License Agreement.

17. Despite demands by AntiCancer, Pfizer has failed to pay the annual maintenance fee that was due no later than January 6, 2011.

18. Section 9.5 of the License Agreement provides that upon termination by the Licensee under Section 9.2, "all rights and licenses granted to Licensee hereunder shall immediately terminate and all rights to the Licensed Technology shall revert to Licensor." Under the License Agreement "Licensed Technology" includes the rights, *inter alia*, to the Know-How, Cell Lines, and Animal Models provided by AntiCancer.

19. Pfizer has not returned to AntiCancer any of the Know-How, Cell Lines, or Animal Models, as required upon termination of the License Agreement.

PATENT INFRINGEMENT FACTS

20. AntiCancer is a world leader in experimental animal models of cancer and other diseases. Among AntiCancer's patented suite of technology are methods for tracking the expression of specific genes in animals through the use of fluorescent proteins, including green fluorescent protein ("GFP"), a protein which occurs naturally in a species of jellyfish. Researchers can track the expression of a gene of interest by viewing the animal or by creating optical images of the animal as it expresses GFP (as well as other fluorophores of various colors, which taken together are known by those skilled in the art as GFP).

21. Another groundbreaking technology developed and patented by AntiCancer is a process known as "surgical orthotopic implantation" (SOI). Researchers using this technology can implant fragments of various types of human tumors onto an organ of a mouse or other rodent which corresponds to the human organ from which the tumor was originally obtained, and conduct experiments related to the resulting growth of the tumor within the mouse, including drug discovery and efficacy experiments. This method can also be used in conjunction with AntiCancer's GFP-related imaging technology.

22. In August 2009, the journal *Biochemical and Biophysical Research Communications* published a paper titled "Defects in embryonic development of EGLN1/PHD2 knockdown transgenic mice are associated with induction of Igfbp in the placenta" (the "Defects in embryonic development" paper, a true and correct copy of which

is attached hereto and incorporated by reference as Exhibit “3”). Four out of five of the authors of the paper, including its corresponding author, are Pfizer scientists. The paper describes a study in which GFP expression in mouse embryos was monitored and non-invasively imaged.

23. In November 2009 at the 20th Asia Pacific Cancer Conference, scientists from both Pfizer and Crown made a joint poster presentation titled “Establishment of the Orthotopic Primary Human Hepatocellelar Carcinoma Model for Oncology Drug Discovery” (the “Establishment of the Orthotopic Primary Human Hepatocellelar Carcinoma Model” poster, a true and correct copy of which is attached hereto and incorporated by reference as Exhibit “4”). The poster describes how the Pfizer and Crown scientists surgically orthotopically implanted tumor fragments from patient liver-tumor tissues into the liver of mice, and then treated them with a drug called sunitinib malate (Sutent), which is marketed by Pfizer. The type of cancer studied, Hepatocellular carcinoma (HCC), is described by the Pfizer and Crown scientists as having “[m]ost cases occur in Asia.” (Exhibit 4.)

24. On December 8, 2009, Pfizer and Crown issued a joint press release announcing “A Collaboration To Research And Develop New Treatments For Asian Cancers” (the “Pfizer-Crown Collaboration” press release, a true and correct copy of which is attached hereto and incorporated by reference as Exhibit “5”). The press release quotes Alex Wu, the CEO of Crown, who states that “[t]his new collaboration extends an already very successful partnership between Crown and Pfizer.”

<u>FIRST CLAIM FOR RELIEF</u>

(Breach of Contract – License Agreement)

(Against Pfizer)

25. AntiCancer realleges and incorporates by reference as though fully set forth preceding paragraphs 1 through 24.

26. There exists a valid technology licensing agreement between AntiCancer and Pfizer, dated and effective December 22, 2006 (the “License Agreement”).

27. AntiCancer has performed all conditions, covenants, and promises required by it on its part to be performed in accordance with the terms and conditions of the License Agreement.

28. Pfizer has breached the License Agreement by failing to pay the annual maintenance fee of $150,000 due no later than January 6, 2011.

29. As a direct and proximate result of Pfizer's failure to pay the maintenance fee as required under the License Agreement, AntiCancer has been damaged in the amount of at least $150,000.

SECOND CLAIM FOR RELIEF

(Breach of Implied Covenant of Good Faith and Fair Dealing)

(Against Pfizer)

30. AntiCancer realleges and incorporates by reference as though fully set forth preceding paragraphs 1 through 29.

31. Implied in the License Agreement is a covenant of good faith and fair dealing.

32. Pfizer has breached the implied covenant of good faith and fair dealing by attempting to avoid the notice requirements of the termination rights contained in the License Agreement through a bad faith and fraudulent backdating of its purported termination notice.

33. Pfizer's breach of the implied covenant of good faith and fair dealing was intentional and made with the specific purpose of unfairly and improperly withholding the benefits due to AntiCancer under the License Agreement.

34. As a direct and proximate result of Pfizer's breach of the covenant of good faith and fair dealing, AntiCancer has been damaged in an amount to be proven at trial, in addition to attorneys fees and expenses.

THIRD CLAIM FOR RELIEF

(Unjust Enrichment)

(Against Pfizer)

35. AntiCancer realleges and incorporates by reference as though fully set forth preceding paragraphs 1 through 34.

36. Pfizer has obtained valuable intellectual and physical property from AntiCancer, including but not limited to, Know-How, Cell Lines, and Animal Models (the "Licensed Technology").

37. Pfizer has obtained the Licensed Technology at AntiCancer's expense and efforts, and as a result of AntiCancer's good faith performance under the License Agreement.

38. Upon Pfizer's termination of the License Agreement, any and all of Pfizer's rights to the Licensed Technology terminated and all rights to the Licensed Technology reverted back to AntiCancer.

39. Pfizer has not returned any of the Licensed Technology or made monetary restitution of its value. Pfizer's unjustified and continuing detention of the Licensed Technology constitutes unjust enrichment, and the circumstances are such that in equity and good conscience Pfizer should return the Licensed Technology or make monetary restitution of its value, in an amount to be proven at trial.

FOURTH CLAIM FOR RELIEF

(Infringement of '159 Patent)

(Against Pfizer)

40. Plaintiff realleges and incorporates by reference as though fully set forth preceding paragraphs 1 through 39.

41. United States Patent No. 6,649,159 (the "'159 Patent") issued on November 18, 2003. A true and correct copy of the '159 Patent is attached hereto as Exhibit "6" and incorporated herein by this reference.

42. Plaintiff is the sole owner of the '159 Patent.

43. Plaintiff is informed and believes that Pfizer has infringed, and still is infringing, the '159 patent by making, using, selling, offering for sale and/or licensing products and services covered by one or more claims of the '159 Patent, including but not limited to, through the activities described in the "Defects in embryonic development" paper (Exhibit 3), without plaintiff's authorization or consent.

44. Plaintiff is informed and believes that Pfizer has infringed the '159 Patent, and will continue to do so unless enjoined by this Court.

45. Plaintiff is informed and believes, and on that basis, alleges that Pfizer is aware of the '159 Patent and that its infringement has been willful.

46. By reason of the foregoing, plaintiff has suffered damages in an amount to be proven at trial and, in addition, has suffered irreparable loss and injury.

47. The acts of infringement described above are willful, deliberate and in reckless disregard of plaintiff's patent rights.

FIFTH CLAIM FOR RELIEF

(Infringement of '812 Patent)

(Against Pfizer and Crown)

48. Plaintiff realleges and incorporates by reference as though fully set forth preceding paragraphs 1 through 47.

49. United States Patent No. 5,569,812 issued on October 29, 1996, and reissued as RE 39,337E on October 10, 2006 (the "'812 Patent"). A true and correct copy of the '812 Patent (including RE 39,337E) is attached hereto as Exhibit "7" and incorporated herein by this reference.

50. Plaintiff is the sole owner of the '812 Patent.

51. Plaintiff is informed and believes that both defendants have jointly and severally infringed, and still are infringing, the '812 patent by making, using, selling, offering for sale and/or licensing products and services covered by one or more claims of the '812 Patent, including but not limited to, through the activities described in the "Establishment of the Orthotopic Primary Human Hepatocellelar Carcinoma Model" poster (Exhibit 4), without plaintiff's authorization or consent.

52. Plaintiff is informed and believes that defendants have infringed the '812 Patent and will continue to do so unless enjoined by this Court.

53. Plaintiff is informed and believes, and on that basis, alleges that defendants are aware of the '812 Patent and that their infringement has been willful.

54. By reason of the foregoing, plaintiff has suffered damages in an amount to be proven at trial and, in addition, has suffered irreparable loss and injury.

55. The acts of infringement described above are willful, deliberate and in reckless disregard of plaintiff's patent rights.

PRAYER FOR RELIEF

WHEREFORE, Plaintiff AntiCancer prays for relief as follows:

(1) That the Court order Pfizer to pay AntiCancer's direct damages for breach of contract in the amount of $150,000 and indirect damages according to proof at trial;

(2) That the Court order Pfizer to return all wrongfully detained property of AntiCancer or award monetary restitution of the value thereof;

(3) That defendant Pfizer be adjudged to have willfully infringed the '159 patent under 35 U.S.C. § 271(a), (b), (c), and (g);

(4) That all defendants, and each of them, be adjudged to have willfully infringed the '812 patent under 35 U.S.C. § 271(a), (b), (c), and (g);

(5) That the Court award AntiCancer enhanced damages, and defendants' profits, pursuant to 35 U.S.C. § 284, for defendants' willful infringement of the '159 and '812 patents;

(6) That defendants, and each of them, be preliminarily and permanently restrained and enjoined under 35 U.S.C. § 283 from directly or indirectly infringing the '159, and/or '812 patent(s);

(7) That the Court award AntiCancer its attorney's fees pursuant to 35 U.S.C. § 285 incurred by it in prosecuting this action;

(8) That the Court assess pre-judgment and post-judgment interest and costs of suit against defendants, and award such interest and costs to AntiCancer; and,

(9) That AntiCancer have such other and further relief as this Court may deem just and proper.

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Respectfully submitted,

Dated: October 4, 2011

ANTICANCER, INC.

By: s/Matt Valenti
Matt Valenti
Attorney for Plaintiff ANTICANCER, INC.

DEMAND FOR TRIAL BY JURY

AntiCancer hereby demands a trial by jury as to all issues triable by jury.

Respectfully submitted,

Dated: October 4, 2011 ANTICANCER, INC.

By: s/Matt Valenti
Matt Valenti
Attorney for Plaintiff ANTICANCER, INC.

EXHIBIT 1

EXHIBIT 1

From: Rosado, Beth [mailto:Beth.Rosado@pfizer.com]
Sent: Wednesday, December 08, 2010 10:41 AM
To: all@anticancer.com
Subject: Termination letter

I am in the process of drafting a termination letter for a license agreement between Wyeth (now Pfizer, Inc.) and AntiCancer, Inc dated December 22, 2006.

Can someone please verify whether there is someone in addition to Dr. Hoffman who should receive the letter?

Thank you
Beth

Beth E. Rosado
CIBB
BioTherapeutics R&D
Pfizer, Inc.
401 N. Middletown Rd., 200/4502
Pearl River, NY 10965
P: (845) 602-4568
F: (845) 474-3264
beth.rosado@pfizer.com
please don't print this e-mail unless necessary

EXHIBIT 2

EXHIBIT 2

From: "Rosado, Beth" <Beth.Rosado@pfizer.com>
To: "AntiCancer, Inc." <team.acct@yahoo.com>
Sent: Wed, December 15, 2010 8:56:46 AM
Subject: RE:

Please see the attached termination letter for the License agreement with AntiCancer. A copy is being sent on to Dr. Robert Hoffman.

Thank you,
Beth

please don't print this e-mail unless necessary

From: AntiCancer, Inc. [mailto:team.acct@yahoo.com]
Sent: Thursday, December 09, 2010 1:26 PM
To: Widbin, Karen
Cc: Rosado, Beth
Subject:

Dear Customer :

Please find attached the invoice for the annual renewal of our license agreement. We would appreciate payment by 12/22/2010.

Thanks very much,

AntiCancer, Inc.
858-654-2555

To view your invoice
Open the attached PDF file. You must have Acrobat® Reader® installed to view the attachment.

Beth E. Rosado
Operations Associate
401 N. Middletown Road
Pearl River, NY 10965
845-602-4568 tel.
845-474-3264 fax
Beth.Rosado@pfizer.com

Pfizer Inc
235 East 42nd Street
New York, NY 10017

December 8, 2010

Robert M. Hoffman, Ph.D.
President
AntiCancer, Inc.
7917 Ostrow Street
San Diego, CA 92110

Re: Termination of License Agreement between Pfizer Inc and AntiCancer dated December 22, 2006 (the Agreement")

Dear Dr. Hoffman:

It has been determined that the license granted to Pfizer under the Agreement is no longer needed. Pursuant to Section 9.2 of the Agreement, Pfizer hereby gives notice of termination of the Agreement. The Agreement shall therefore terminate on December 8, 2010. If there are any questions or concerns, please contact Beth Rosado.

Regards,

Robert T. Abraham

Robert T. Abraham, Ph.D.
VP and CSO, Center for Integrative Biology and Biotherapeutics
Pfizer Inc

EXHIBIT 3

EXHIBIT 3

Biochemical and Biophysical Research Communications 390 (2009) 372–376

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Defects in embryonic development of EGLN1/PHD2 knockdown transgenic mice are associated with induction of Igfbp in the placenta

Terence R.S. Ozolinš [a], Timothy S. Fisher [b], Diane M. Nadeau [c], Jeffrey L. Stock [c], Anne S. Klein [d], Anthony J. Milici [d], Daniel Morton [e], Margaret B. Wilhelms [e], William H. Brissette [b], Baiyong Li [b,*]

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[b] Department of Antibacterials, Immunology and Cancer, Pfizer Global Research and Development, Groton, CT 06340, USA
[c] Genetically Modified Mice Center of Emphasis, Pfizer Global Research and Development, Groton, CT 06340, USA
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ARTICLE INFO

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ABSTRACT

The HIF (hypoxia inducible factor) hydroxylases EGNL1/PHD2 has been implicated in embryonic development. Here we knocked down EGNL1 in vivo by injecting one-cell murine zygotes with lentivirus-containing RNAi. Progeny with demonstrated EGLN1 inhibition had elevated EPO production and erythropoiesis in vivo. The partial inhibition of EGLN1 in utero is embryonic lethal in some, but not all mice on gestation day 14, and is associated with defects in placental and heart development, similar to those noted in the EGLN1 knockout mouse. Importantly, the in utero inhibition of EGNL1 varied greatly between the embryo proper and the placenta. Using this as a tool we show that the embryopathic effects are associated with knockdown of EGNL1 and the associated induction of Igfbp1 (insulin-like growth factor binding protein-1) mRNA in the placenta, but not the embryo.

Eukaryotic cells adapt to hypoxic conditions by inducing the expression of a number of genes that mobilize appropriate cellular response mechanisms. These genes include EPO which promotes hematopoiesis, VEGF which stimulates angiogenesis, and genes involved in glucose transport and metabolism [1]. Such responses serve to optimize tissue utilization of oxygen and nutrients in a low oxygen environment. The key transcription factor coordinating the induction of genes in the hypoxia response is HIF [2,3].

HIF-1 is composed of two subunits: the hypoxia-regulated subunit, HIF-1α, and the oxygen-insensitive HIF-1β subunit [4]. Under normoxia, the HIF-1α subunit is rapidly degraded via the von Hippel–Lindau tumor suppressor gene product (pVHL)-mediated ubiquitin–proteasome pathway [5–8]. The association of pVHL and HIF-1α under normoxic conditions is triggered by the post-translational hydroxylation of several prolines within a polypeptide segment known as the oxygen-dependent degradation (ODD) domain [9–11] through the function of specific HIF-prolyl hydroxylases [12–14]. The hydroxylation of an asparagine in the C-terminal transactivation domain (C-TAD) of HIF-1α inhibits interaction with the p300/CBP coactivator and reduces the transcriptional activity of HIF-1 during normoxic conditions [15–17].

EGLN1 knockout mice die in utero and conditional knockout of EGLN1 led to hyperactive angiogenesis and angiectasia [18]. Here we generated transgenic mice expressing EGLN1 shRNA to further examine the mechanism underlying the developmental defects associated with EGLN1 inhibition.

Materials and methods

Transgenic mice. Lentivirus harboring EGLN1 shRNA was microinjected into the perivitelline space of mouse zygotes. The zygotes were implanted into pseudopregnant female mice to generate transgenic mice. Following weaning of litters generated from injected embryos, tail snips were collected from the transgenic litters and analyzed by genomic PCR and by fluorescence microscopy to establish a subjective scale of relative GFP expression. Animals were sacrificed at varying ages and compared to age and sex matched normal controls. Tissue samples were collected into RNA Later solution and stored at 4 °C prior to RNA purification.

Histology. Kidney samples were removed at necropsy and immersion fixed for 1 h in 4% paraformaldehyde. The samples were processed and frozen in OCT. Five micron cryosections were cut and stored at −20 °C. Sections were warmed to room temperature,

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E-mail address: Baiyong.Li@pfizer.com (B. Li).

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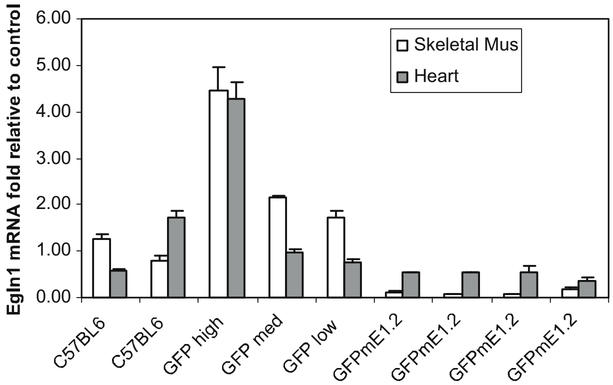


Fig. 1. (A) Knockdown of EGLN1 mRNA in muscle tissues in transgenic mice. RNA was extracted from skeletal and heart muscle of adult mice expressing either GFP alone or in combination with EGLN1 shRNA E1.2. The level of EGLN1 expression was examined using real-time qPCR.

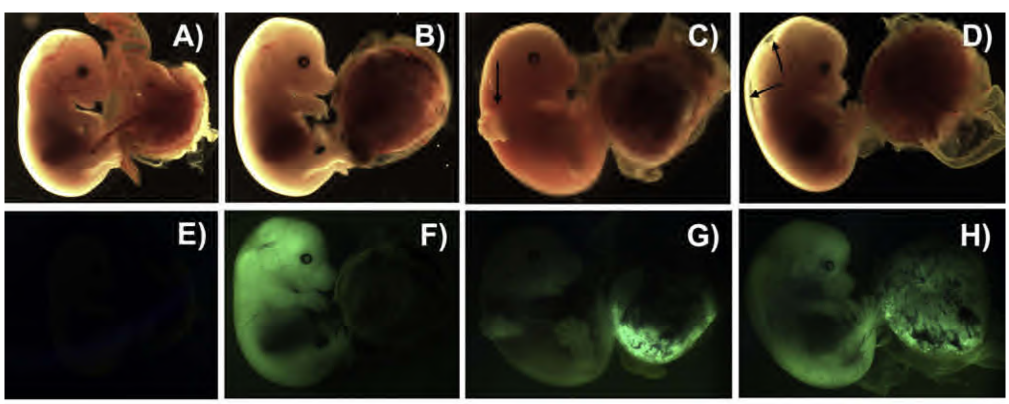
washed in TBS (Tris-buffered saline with 25 mM Tris, 0.15 M NaCl, pH 7.5) fixed in acetone (4 min at 4 °C), rinsed in TBS and coverslipped. All sections were evaluated for Green Fluorescent Protein (GFP) fluorescence on a Nikon E800 microscope.

Mouse embryos and placentas were removed and placed into 10% neutral buffered formalin for fixation for at least 48 h. Placentas were detached from the embryos and both were placed into cassettes separately for processing. The tissues were processed overnight through alcohol and xylene into paraffin using a Shandon® Pathcentre Tissue Processor. Embryos and placentas were embedded in Paraplast Plus™—embryos oriented in a frontal plane for cutting back to front, placentas oriented on edge for cross-section cutting. The placenta tissue blocks were cut on a microtome at 5 μm—one full section through the center was obtained and collected onto a glass slide. Embryos were also cut on a microtome at 5 μm but once the level of the heart was reached every section was saved on a glass slide. The slides were stained with hematoxylin and eosin on a Shandon® automatic slide stainer.

qRT-PCR analysis of tissue samples. Tissue or embryo samples were collected into RNA later solution and stored at 4 °C prior to processing. Tissue samples were homogenized in 1 ml Trizol reagent using 5 mm steel beads in 1.5 ml tubes. Each tube was subjected to mixing at 25 Hz for 5 min using a Qiagen Tissuelyser. mRNA was generated as described above and cDNA was used as template in qPCRs using ABI pre-designed assay reagents. Relative target mRNA expression was calculated using the ddCt method utilizing β-actin as the normalizing factor and related to associated controls.

Results and discussion

To examine the developmental effect of EGLN1 we generated transgenic mice expressing EGLN1 shRNA. We modified a previously described lentiviral vector [19] to drive shRNA expression under a ubiquitously expressed H1 promoter replacing the original U6 promoter. The accompanying paper titled "Analysis of the role of the HIF hydroxylase family members in erythropoiesis" describes the generation of the EGLN1 RNAi transgenic mice in detail. When examining the level of knockdown of EGLN1 gene in the transgenic mice, we observed significant knockdown (similar to in vitro levels) in skeletal muscle but not in heart (Fig. 1A) or kidney (data not shown). A closer examination of the kidney of the transgenic mice showed variegated expression of GFP (data not shown). This is consistent with previous observation of variegated expression of this vector in transgenic mice [20]. Therefore we reasoned that in the kidney the knockdown effect at the level of mRNA and protein assessment was "masked" by a high proportion of cells within the analysis sample not expressing the hairpin while at the



Embryoplacental effects of the localization of intense (+++) GFP fluorescence in EGNL1 RNAi hairpin treated embryos				
Location of Fluorescence	Head length (mm)	Crown rump Length (mm)	Placental weight (mg)	N
None	7.08 ± 0.18	12.39 ± 0.85	105.1 ± 12.1	13
Embryo only	6.95 ± 0.26	11.88 ± 1.36	111.8 ± 10.5	4
Placenta only	6.37 ± 0.41	10.23 ± 0.31	129.5 ± 13.5	4
Placenta and Embryo	6.35 ± 0.20	10.45 ± 1.21	123.5 ± 9.7	5

Fig. 2. The embryoplacental effects of the localization of intense (+++) GFP fluorescence in EGNL1 RNAi hairpin treated embryos on E14.5. The fluorescent intensities were graded as 0, +, ++, or +++, and the conceptuses divided into four groups; no fluorescence, intense (+++) fluorescence only in the embryo, the placenta, or both embryo and placenta. Representative embryos are depicted in dark field (A–D) or under fluorescent light (E–H). No malformations are apparent with in the absence of a GFP signal (A,E) or if the GFP signal is only noted in the embryo proper (B,F). Poor limb development (C,G; arrow) and edema in (D,H; arrows) were noted in embryos with intense placental GFP fluorescence. The faint embryonic glow in G is reflected light only. Head length and crown rump length trended lower and placental weights increased with GFP detected in the placenta. Fluorescence only in the embryo had no effect on embryoplacental parameters.

functional level enough cells were generating the RNAi molecule to manifest the increased EPO phenotype. To examine the broader effect of EGLN1 inhibition on gene expression, we used a plate based real-time PCR system (the hypoxia Superarray) to survey the expression of hypoxia-related genes in the kidney samples of transgenic mice. Remarkably, among 84 genes related to hypoxia, EPO was the only gene with a statistically significant increase (34-fold increase). The remaining genes represented on the assay, including VEGF, showed no significant changes (data not shown).

We next examined the effects of EGLN1 knockdown during embryogenesis. The number of viable births in surrogate dams carrying embryos treated with the EGLN1 RNAi hairpin was consistently lower than in those carrying the control luciferase hairpin, suggesting expression of the EGLN1 hairpin may be embryonic lethal (data not shown). Indeed, EGLN1 $^{-/-}$ embryos die in utero between E12.5 and E14.5 [18], prompting us to examine EGLN1 RNAi transgenic embryos at a single time point during gestation. On E14.5, the viability of control virus treated embryos and EGLN1 RNAi virus treated embryos was 85% and 67%, respectively. Moreover, in the control group, late resorptions (dead with distinguishable embryonic form) were not GFP positive, whereas in contrast, virtually all EGLN1 RNAi virus treated late resorptions were strongly GFP positive, further confirming the lethality of the EGLN1 RNAi hairpin in some, but not all embryos.

The localization and intensity of GFP fluorescence in conceptuses from both treatment groups was varied. To determine whether the location and presumptive degree of EGLN1 knockdown influenced developmental outcome, the location and intensity (arbitrarily graded as 0, +, ++, or +++) of the fluorescence was noted. Gross anomalies including poor limb development and edema were only seen in embryos in which strong placental fluorescence was noted, alone or in conjunction with strong embryonic fluorescence (Fig. 2C and D). Placental fluorescence (+++), alone or with embryo fluorescence (+++), was correlated with a trend toward a decrease in head length, crown rump length, and an increase in placental weight (Fig. 2). Although not statistically significant due to small sample size, we believe these changes are biologically relevant. Taken together, the malformations and growth effects suggest that, at this stage of gestation, it is the placental knockdown of EGNL1, and not the embryonic knockdown, that determines the developmental outcome.

In a separate cohort, real-time qPCR analysis was used to identify embryos with a significant reduction of EGLN1 mRNA to further characterize resultant changes in downstream gene expression and tissue histology. Surprisingly, using hypoxia arrays, the knockdown of EGLN 1 only altered the mRNA expression of a few genes. Most significant was that the tissue-specific knockdown of EGLN1 was correlated with a significant increase in mean insulin-like growth factor binding protein-1 (Igfbp1) mRNA levels in both the fetal liver and placenta of the E14.5 embryo compared to vector control embryos (Fig. 3A–D). No other significant changes were noted in the placenta (data not shown), and in the fetal liver,

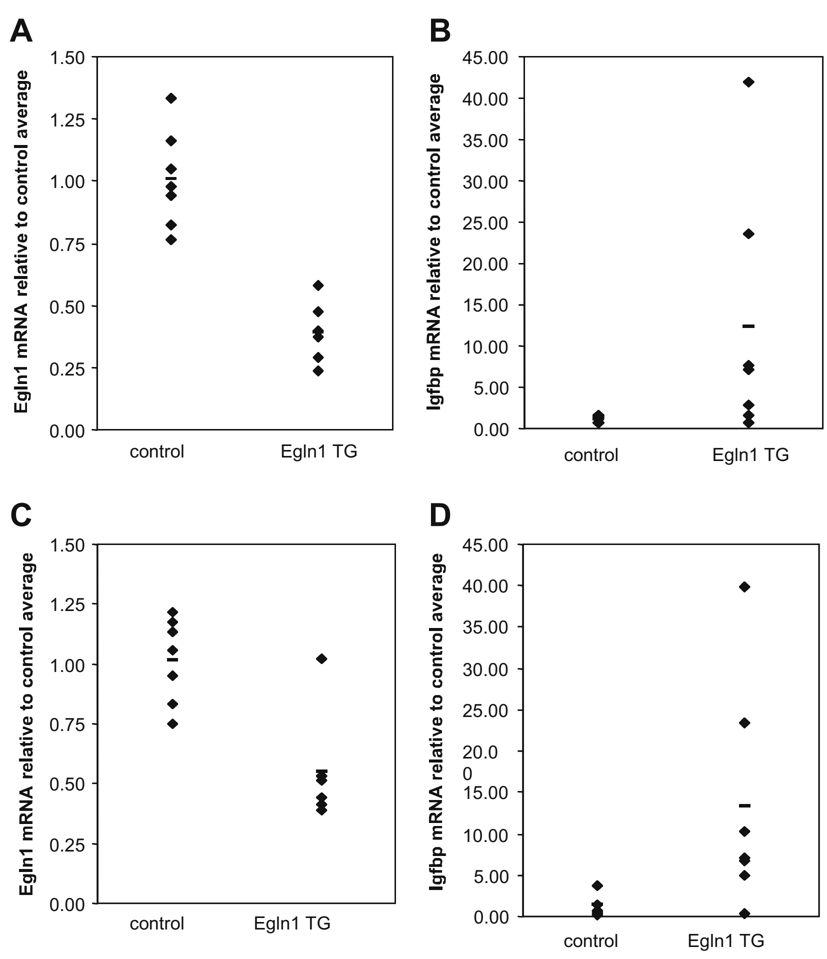


Fig. 3. Knockdown of EGLN1 and stimulation of Igfbp1 expression in the developing embryo of transgenic mice. E14.5 embryos of transgenic mice expressing shRNA E1.2 were examined using real-time qPCR. Age and sex matched transgenic mice expressing GFP alone were used as controls in all experiments. (A) EGLN1 knockdown in the fetal liver. (B) Increases in Igfbp1 mRNA level in the fetal liver in EGLN1 RNAi mice. (C) EGLN1 knockdown in the placenta. EGLN1 knockdown in the fetal liver. (D) Igfbp1 mRNA level in the placenta.

only Bhlhb2 was increased, but importantly VEGF was not (data not shown).

To determine the histologic consequences of EGNL1 knockdown and associated Igfbp1 induction, control vector embryos were compared to embryos with confirmed reduction of EGNL1 mRNA (mean of 47 ± 25% of control levels). Decreased levels of EGLN1mRNA were correlated with histological differences in placenta and embryonic liver and heart. In placenta, knockdown was associated with a gross disruption of the normal architecture including empty spaces in the labyrinth, relative reduction in connective tissue of the labyrinth, and a relative increase in the proportion of trophoblastic cells and necrotic cells. More specifically, spongiotrophoblasts extended into the labyrinth (Fig. 4A and D) and there were decreases in the number of very large trophoblast giant cells compared to control embryos (Fig. 4B and E). These microscopic findings would not be expected to compromise fetal survival. Liver abnormalities were noted in most knockdown embryos, including a decreased numbers of large immature cells (hepatocellular and/or hematopoietic precursors) with more maturing erythroid cells (Fig. 4C and F), and in one instance, the partial replacement of the cellular components of the liver by blood-filled spaces and necrotic debris.

Six of eight knockdown hearts had microscopic abnormalities including poorly defined ventricular chambers, increased numbers of myocardial cell trabeculae surrounded by small luminal blood spaces (compare inset Fig. 4G and H) and mild ventricular dilatation. The ventricular and septal walls were also thinner (compare Fig. 4G and I) with one instance of an interventricular septum that would permit blood to flow between ventricles Fig. 4I).

Here, using an RNAi hairpin approach, we confirmed that EGLN1 is critical for embryogenesis. The effects of EGLN1 knockdown included embryo lethality, intrauterine growth retardation, structural defects in the placental labyrinth, underdeveloped heart trabeculae, and ventricular septal defects. This recapitulated the phenotype noted in EGLN1 $^{-/-}$ embryos [18] although the severity in the current model was variable between embryos. This is likely the result of differences in the amount of EGLN1 knockdown, its gross tissue localization, and variegated hairpin expression within the tissues.

One consequence of EGLN1 knockdown was the partial induction of the hypoxia-induced gene expression response in embryonic liver and placenta, manifest primarily by the induction of Igfbp1 mRNA. At least five different transgenic mouse models over expressing Igfbp1 have been generated [21–25], and in those where embryo/fetal development was specifically examined, a recurring observation was embryolethality [22,23] and reduced embryo/fetal growth [21–23,25]. It has been suggested the excess Igfbp1 may bind IGF, thereby reducing its bioavailability and inhibiting its in utero mitotic effects. The Igfbp1-mediated growth inhibition appears to be evolutionarily conserved as it has also been noted in a zebrafish embryo hypoxia model [26]. Taken together, this suggests that the etiology of the current EGLN1 knockdown phenotype and the previously reported EGLN1 knockout phenotype [18] is likely the consequence of the downstream Igfbp1 induction.

Although EGLN1 knockdown was confirmed in limb bud, liver and placenta, several factors lead us to suggest that the placental is the primary site of action. First, the most striking trends in the inhibition of embryonic growth were only seen with presumptive knockdown in the placenta, not the embryo proper. Second, gross anomalies such as improper limb development and edema occurred with intense fluorescence of the placenta, either alone or in conjunction with embryonic fluorescence, but not with embryonic fluorescence alone. Lastly, a transgenic mouse model with the over expression of human Igfbp1 in the placenta driven by a

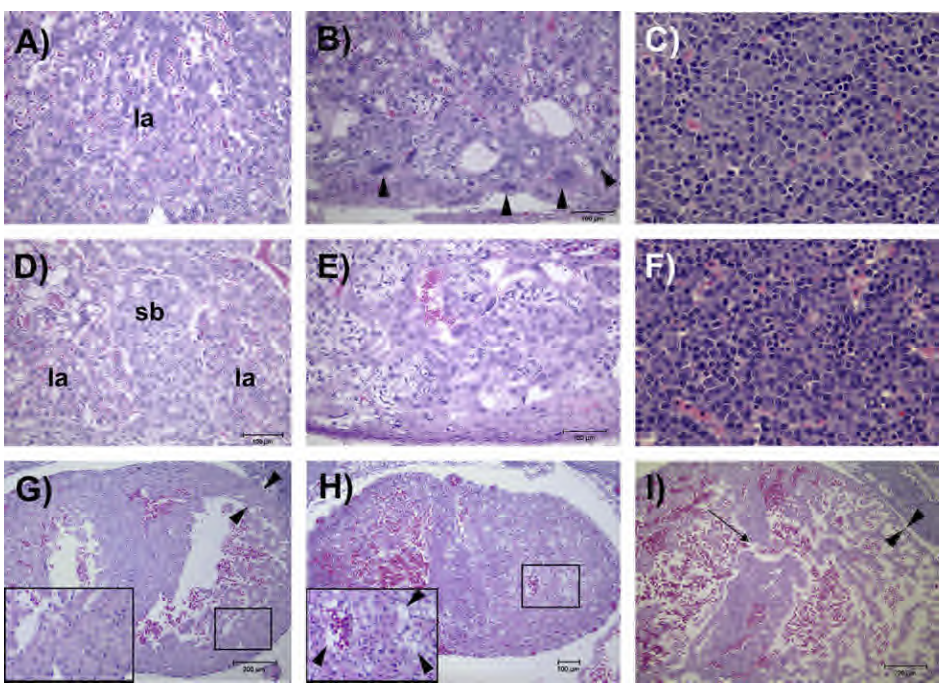


Fig. 4. The histologic consequences of confirmed knockdown of EGNL1 (47 ± 25% control levels). Embryos were treated with control luciferase hairpin (A, B, C, and G) or EGLN1 RNAi hairpin (D, E, F, H, and I), and knockdown of EGNL1 confirmed by qRT-PCR on E14.5. In placenta, knockdown was associated with disruption of the normal architecture including empty spaces and reduced vascularization of the labyrinth. In several instances, spongiotrophoblasts (sp) extended inappropriately into the labyrinth (la) (D). Compared with controls (B), the numbers of very large trophoblast giant cells (arrowheads) were reduced after EGNL knockdown (E). In the liver, knockdown embryos (F) had decreased numbers of large immature cells (hepatocellular and/or hematopoietic precursors) compared to control (C). Hearts had microscopic abnormalities including poorly defined ventricular chambers (H), and increased numbers of myocardial cell trabeculae surrounded by small luminal blood spaces (compare inset G and H). The ventricular and septal walls were also thinner (compare arrowheads G and I) with one instance a ventricular septal defect that would permit blood to flow between ventricles (arrow; I).

native promoter produced placental histologic findings and embryonic growth retardation similar to what we report here [22].

There are several reasons why the importance of placental Igfbp1 levels may supersede those of the fetal liver in determining embryonic outcome. First, Igfbp1 is not expressed in normal mouse placenta and yolk sac [27], suggesting more severe consequences as a result of induced expression. This contrasts with the liver and other tissues of the embryo in which Igfbp1 is basally expressed [25]. Second, in a mouse model over expressing human Igfbp1 in the placental decidua and fetal liver (after E14.5), hIgfbp1 can only be detected in amniotic fluid of embryos carried by transgenic females [22]. This implies that Igfbp1 is derived from maternal decidua. Moreover, in that model amniotic hIgfbp1 levels were independent of fetal genotype, suggesting that Igfbp1 produced in the liver and elsewhere in the embryo proper may act locally, in a paracrine or autocrine manner. Our observations that the presumptive placental knockdown of EGNL1 mediated the embryo growth retardation and increased placental weight independent of presumptive embryonic knockdown are consistent with these previous reports. Furthermore, our data suggest the previously reported placental effects of the homozygous EGNL1 knockout were mediated by the in utero inhibition of IGF activity by excess Igfbp1.

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EXHIBIT 4

EXHIBIT 4

ESTABLISHMENT OF THE ORTHOTOPIC PRIMARY HUMAN HEPATOCELLULAR CARCINOMA MODEL FOR ONCOLOGY DRUG DISCOVERY

Gang Gary Li[1], Wenwei Li[2], Dawei Chen[2], Yiyou Chen[2], James Christensen[1], David Pocalyko[1]

[1]*Oncology, Pfizer, USA,* [2]*CrownBio*

Introduction: Hepatocellular carcinoma (HCC) is the most common liver malignancy, and the third most common cause of cancer-related mortalities worldwide. Most cases occur in Asia, and in China alone an estimated 251,000 male and 95,000 female patients are diagnosed annually. Although significant advances have been made with the use of certain chemotherapeutic and targeted therapeutic agents in HCC treatment, the prognosis for most liver cancer patients remains poor. Pfizer Oncology has significantly strengthened its research and drug discovery activities targeting HCC. As part of the effort, we have developed series of animal models that are instrumental in target validation, efficacy screening and translation research.

Methods: Tumor fragments derived from patient tumor tissues were surgically implanted into the left lobe of nude mouse liver. Tumor-bearing mice were treated with sunitinib malate (Sutent) or vehicle control from day 7 post implantation for 3 weeks. Plasma samples were collected at different time points for alpha-feto-protein (AFP) measurement. At termination, tumors were excised from liver and their weights and sizes were recorded.

Results: Sutent was well tolerated in the animals during the course of the study. Sutent treatment significantly inhibited orthotopic HCC tumor growth measured by size, weight and circulating AFP. In addition, histological analysis confirmed that orthotopically implanted primary human tumors maintained their histopathological characteristics.

Conclusions: The initial results indicate that this model can better reflect human disease by preserving heterotypic nature of tumor cells and stroma, and by maintaining tissue-specific histopathology and by retaining particular genetic mutations, pathogens and disease markers that are naturally associated with clinical disease, and therefore can serve as a valuable tool for target validation, efficacy study, as well as patient population selection.

Abstract >> Free Paper >> Poster Session >> Poster Presentation 4 (Basic 1)

Poster Presentation 4 (Basic 1)

P-37 BLOCKAGE OF ABCC1/MPR1 EXPRESSION ENHANCED CHEMOTHERAPEUTIC EFFICACY VIA THE MANUPULATION OF NRF2 IN HUMAN ETOPOSIDE-RESISTANT ORAL CANCER CELLS

Huang-Hui Chen, Yen-Ting Cheng, Wen-Yang Lai, Jang-Yang Chang, Ching-Chuan Kuo

National Institute of Cancer Research, National Health Research Institutes, Taiwan

P-38 Kiss-1 suppresses MMP-9 expression by activating with p38 MAP kinase in human stomach cancer

Kyung Hee Lee[1], Eun Young Choi[2], Min Kyoung Kim[2], Byung Ik Jang[3], Tae Nyeun Kim[3], Sang Woon Kim[4], Sun Kyo Song[4], Jae-Ryong Kim[5]

[1]Department of Oncology, Korea, [2]Department of Hematology-Oncology, [3]Gastroenterology, [4]Surgery, [5]Biochemistry and Molecular Biology

P-39 A QUANTITATIVE ANALYSIS OF CpG METHYLATION OF THE TRANSCRIPTION FACTOR 4 GENE IN GASTRIC CARCINOMA USING PYROSEQUENCING

Dong Yi Kim[1], Chan Yong Park[1], Chang Hyun Kim[1], Jae Kyoon Joo[1], Ho Gun Kim[1], Seong Yeob Ryu[1], Young Kyu Park[1], O Jung[1], Jae Hyuk Lee[2], Mi Ran Jung[1]

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P-40 Modulation of Intestinal Epithelial Cell-Derived Cytokine Secretion via Mistletoe Lectin

Suyun Lyu[1], Wonbong Park[2], Chang-Eui Hong[1], Sun-Myung Yun[1], Min-Joo Kim[1], Myung-Chan Cho[1], Ho-Am Jang[1], Seong-Taek Jee[1]

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P-41 ENHANCEMENT OF PROTOPORPHYRIN IX ACCUMULATION BY ALGINATE-INCORPORATED AND FOLIC ACID-CONJUGATED CHITOSAN NANO-PARTICLES FOR COLORECTAL CANCER PHOTODYNAMIC DETECTION

Shu-Jyuan Yang, Kun-Che Tsai, Feng-Huei Lin, Ming-Jium Shieh

Institute of Biomedical Engineering, National Taiwan University, Taiwan

P-42 DETECTION OF EGFR GENE MUTATION AND GENE AMPLIFICATION IN COLORECTAL CANCER

Shanna Chen, Shan-Na Chen, Li-Hui Chow, Tsang-Wu Liu

Division of Cancer Research, National Health Research Institutes, Taiwan

P-43 A comparative study of protein expressions in primary colorectal cancer and synchronous hepatic metastases: The significance of MMP-1 expression

Young Wan Kim[1,2], Youg Taek Ko[1], Nam Kyu Kim[1], Chung Hyun Cheol[3], Byung Soh Min[1], Kang Young Lee[1], Hoguen Kim[4]

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P-44 Cell Death of Colorectal Cancer Stem-Like Cell Was Induced by Photodynamic Therapy with Protoporphyrin IX

Ming-Feng Wei[1], Shiang-You Han[1], Shu-Jyuan Yang[1], Feng-Huei Lin[1], Shih-Chieh Hung[2], Ming-Jium Shieh[1]

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P-45 ESTABLISHMENT OF THE ORTHOTOPIC PRIMARY HUMAN HEPATOCELLULAR CARCINOMA MODEL FOR ONCOLOGY DRUG DISCOVERY

Gang Gray Li[1], Wenwei Li[2], Dawei Chen[2], Yiyou Chen[2], James Christensen[1], David Pocalyko[1]

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YIA P-46 DECREASED EXPRESSION OF NEURENSIN-2 CORRELATES WITH POOR PROGNOSIS IN HEPATOCELLULAR CARCINOMA

Haiqing Ma[1], Xiaoting Liang[1], Jingjing Zhao[1], Hui Wang[1], Jiancong Sun[1], Yibing Chen[1], Ke Pan[1], Minshan Chen[2], Jianchuan Xia[1]

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P-47 WITHDRAWN

P-48 EFFECTS OF AGE ON SUSCEPTIBILITY TO DIETHYLNITROSAMINE-INDUCED HEPATOCARCINOGENESIS IN CONNEXIN 32 DYSFUNCTIONAL TRANSGENIC RATS

Aya Naiki-Ito, Makoto Asamoto, Shugo Suzuki, Shinya Sato, Tomoyuki Shirai

Department of Experimental Pathology and Tumor Biology, Nagoya City University Graduate School of Medical Sciences, Japan

P-49 ROLE OF WERNER SYNDROME PROTEIN IN RESPONSE TO CISPLATIN-INDUCED DNA DAMAGE IN HEPATOCELLULAR CARCINOMA CELLS

Sun-Young Lee[1], Eun-Sun Kim[2], Sojin Park[2], Jin-A Lee[2], Byungchan Ahn[3]

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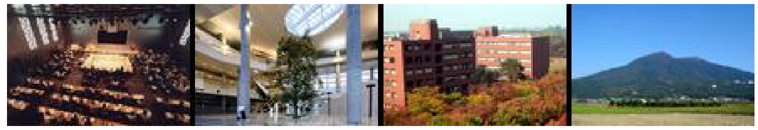

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EXHIBIT 5

EXHIBIT 5

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Crown Bioscience Inc. Tel: 408-986-0103 - Fax: 408-986-0019

Press Release

December 8, 2009

Pfizer

Pfizer And Crown Bioscience Announce A Collaboration To Research And Develop New Treatments For Asian Cancers.

SANTA CLARA, Calif. & NEW YORK --(Business Wire) -- Pfizer (PFE) and Crown Bioscience, Inc. (Crown) announced today that they have entered into a collaboration to research and develop novel therapeutics for Asian cancers. Specific treatments for Asian cancers represent an important unmet medical need as well as a significant market opportunity.

Under the terms of this agreement, Crown will receive an upfront payment and research funding, as well as milestone payments based on the achievement of preclinical and clinical goals.

The companies will work together to discover and advance multiple candidates for clinical development. The work will take place at Crown's new research facility located in Taicang (near Shanghai), China.

> *"I am delighted to be collaborating with Pfizer's exceptional oncology group,"* says Alex Wu, CEO of Crown. *"I am also very happy that Pfizer is focusing on and dedicating resources to address a very important unmet medical need for the Asian populations. This new collaboration extends an already very successful partnership between Crown and Pfizer and further demonstrates Crown's commitment to becoming an outstanding cancer research company in Asia."*

> *"We are delighted to be working closely with Crown to implement a focused drug discovery and development strategy relating to the tumors most prevalent in the Asia region,"* says Neil Gibson, Chief Scientific Officer of Pfizer's Oncology Research Unit. *"By doing so, we believe we can capitalize on the oncology expertise of Crown and enhance our ability to bring novel therapeutics to the marketplace that will benefit cancer patients in Asia."*

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Crown BioScience

Crown Bioscience, Inc. is a U.S.-based drug discovery service company with R&D facilities in China committed to advancing anti-cancer therapeutics through the preclinical and drug development stages. Its proprietary CrystalClear™ and HuPrime™ platforms enable unique lead optimization and translational oncology strategies to deliver better clinical candidates. Our multi-disciplinary team of experts provides an integrated service solution from your idea to your IND, including: preclinical, translational, and clinical capabilities.

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At Pfizer, we apply science and our global resources to improve health and well-being at every stage of life. We strive to set the standard for quality, safety and value in the discovery, development and manufacturing of medicines for people and animals. Our diversified global health care portfolio includes human and animal biologic and small molecule medicines and vaccines, as well as nutritional products and many of the world's best-known consumer products. Every day, Pfizer colleagues work across developed and emerging markets to advance wellness, prevention, treatments and cures that challenge the most feared diseases of our time. Consistent with our responsibility as the world's leading biopharmaceutical company, we also collaborate with health care providers, governments and local communities to support and expand access to reliable, affordable health care around the world. For more than 150 years, Pfizer has worked to make a difference for all who rely on us. To learn more about our commitments, please visit us at www.pfizer.com.

PFIZER DISCLOSURE NOTICE: The information contained in this release is as of December 8, 2009. Pfizer assumes no obligation to update forward-looking statements contained in this release as the result of new information or future events or developments.

This release contains forward-looking information that involves substantial risks and uncertainties about a collaboration between Pfizer and Crown Bioscience, Inc. to research and develop treatments for cancers that are prevalent in Asia. Such risks and uncertainties include, among other things, the uncertainties inherent in research and development; decisions by regulatory authorities regarding whether and when to approve any drug applications that may be filed for any such treatments as well as their decisions regarding labeling and other matters that could affect the availability or commercial potential of such treatments; and competitive developments.

A further description of risks and uncertainties can be found in Pfizer's Annual Report on Form 10-K for the fiscal year ended December 31, 2008 and in its reports on Form 10-Q and Form 8-K.

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EXHIBIT 6

EXHIBIT 6

US006649159B2

(12) **United States Patent**
Yang et al.

(10) **Patent No.: US 6,649,159 B2**
(45) **Date of Patent: Nov. 18, 2003**

(54) **WHOLE-BODY OPTICAL IMAGING OF GENE EXPRESSION AND USES THEREOF**

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(22) Filed: **Mar. 19, 2001**

(65) **Prior Publication Data**

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Related U.S. Application Data

(60) Provisional application No. 60/190,196, filed on Mar. 17, 2000.

(51) **Int. Cl.**^{7} **A61K 48/00**; A61K 49/00; A01N 63/00; C12N 15/63; C12N 15/85

(52) **U.S. Cl.** **424/93.21**; 424/93.1; 424/93.2; 424/9.1; 435/320.1; 435/325

(58) **Field of Search** 800/8; 435/320.1, 435/325; 536/24.5; 424/93.1

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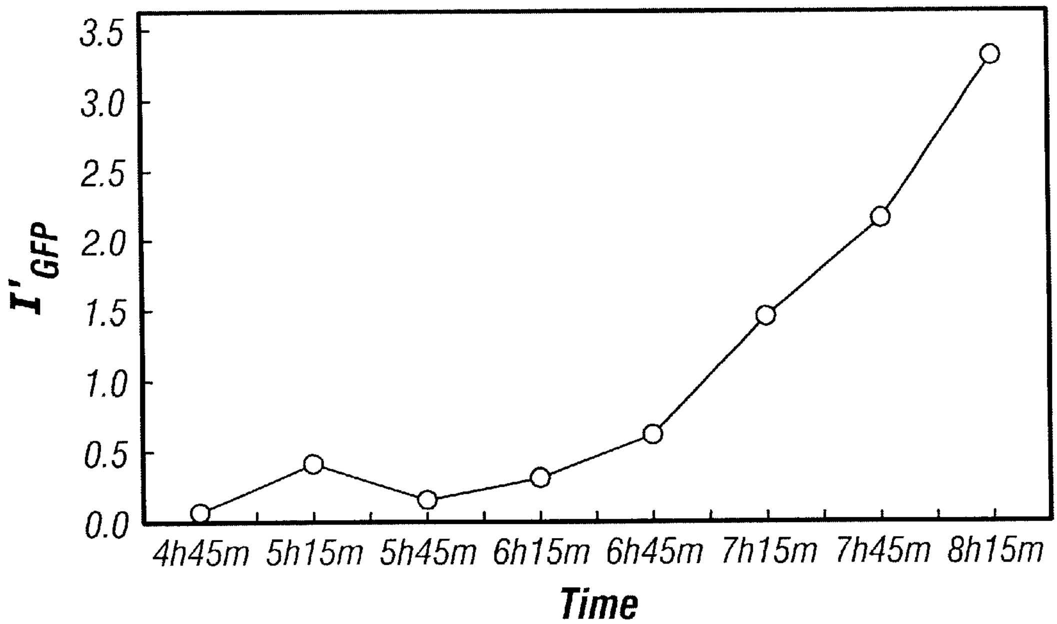
(57) **ABSTRACT**

The invention relates to the whole-body external optical imaging of gene expression. Specifically, methods for whole-body external optical imaging of gene expression and methods for evaluating a candidate protocol or drug for treating diseases or disorders using a fluorophore operatively linked to the promoter of a gene and external optical imaging are provided herein. Methods to screen for substances or genes that regulate target promoters are also provided.

13 Claims, 2 Drawing Sheets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3.0
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2.0
1.5
1.0
0.5
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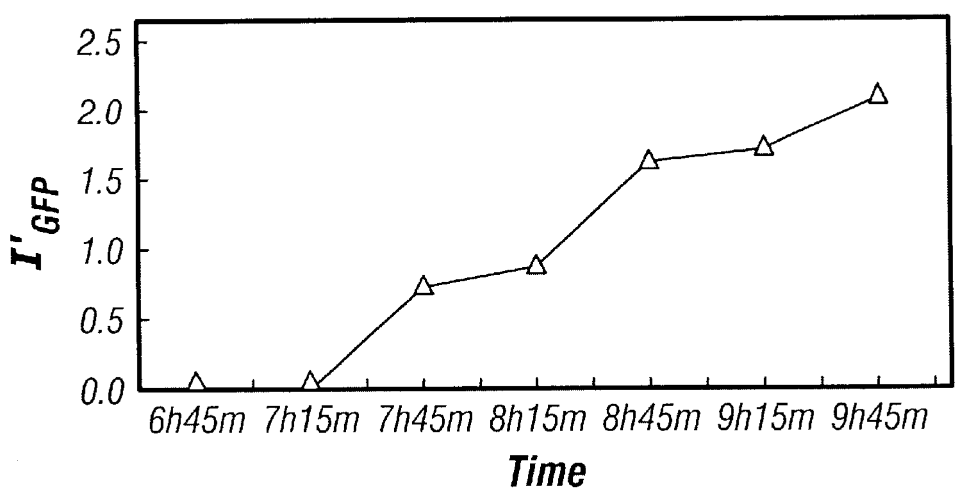
I'_{GFP}
4h45m 5h15m 5h45m 6h15m 6h45m 7h15m 7h45m 8h15m
Time

FIG. 1A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2.0
1.5
1.0
0.5
0.0
I'_{GFP}
6h45m 7h15m 7h45m 8h15m 8h45m 9h15m 9h45m
Time

FIG. 1B

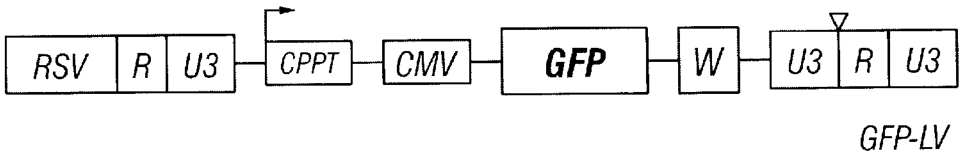
RSV
R
U3
CPPT
CMV
GFP
W
U3
R
U3
GFP-LV

FIG. 2A

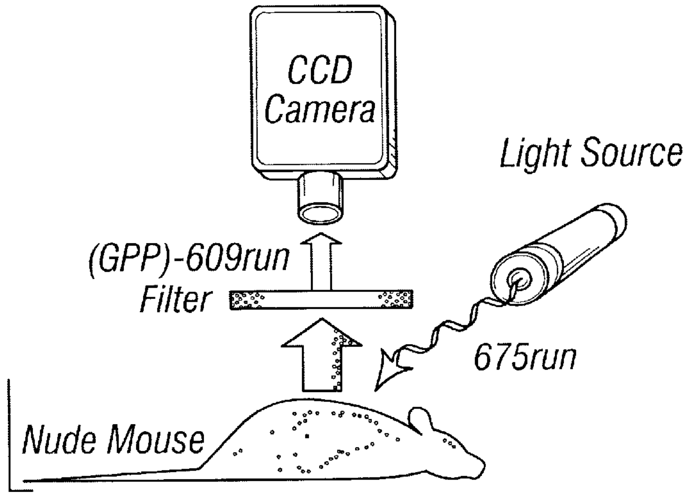
CCD
Camera
Light Source
(GPP)-609run
Filter
675run
Nude Mouse

FIG. 2B

WHOLE-BODY OPTICAL IMAGING OF GENE EXPRESSION AND USES THEREOF

This application claims priority under 35 U.S.C. 119 from provisional application U.S. Ser. No. 60/190,196 filed Mar. 17, 2000, the contents of which are incorporated herein by reference.

TECHNICAL FIELD

The invention relates to the whole-body external optical imaging of gene expression. Specifically, methods for whole-body external optical imaging of gene expression and methods for evaluating a candidate protocol or drug for treating diseases or disorders using a fluorophore operatively linked to the promoter of a gene and external optical imaging are provided herein. Methods to screen for substances or genes that regulate target promoters are also provided.

BACKGROUND ART

Whole-body imaging technology has been used to monitor "tracer molecules" in the intact body. For example, Brenner et al. studied the diagnostic value of iodine-123-2-hydroxy-3-iodo-6-methoxy-N-[(1-ethyl-2-pyrrolidinyl) methyl] benzamide (IBZM) whole-body imaging in comparison to thallium-201 scintigraphy in patients with metastatic malignant melanoma (Brenner et al., *Eur. J. Nucl. Med.,* 26(12):1567–71 (1999)). Benard et al. conducted clinical evaluation of processing techniques for attenuation correction with ^{137}Cs in whole-body PET imaging (Benard et al., *J. Nucl. Med.,* 40(8):1257–63 (1999)). Jerusalem et al. showed that whole-body positron emission tomography using ^{18}F-fluorodeoxyglucose for posttreatment evaluation in Hodgkin's disease and non-Hodgkin's lymphoma has higher diagnostic and prognostic value than classical computed tomography scan imaging (Jerusalem et al., *Blood,* 94(2):429–33 (1999)). Eustace et al. discussed practical issues, clinical applications, and future directions of whole-body MR imaging (Eustace et al., *Magn. Reson. Imaging Clin.* (*N. Am*), 7(2):209–36 (1999)). Engelson et al. studied fat distribution in HIV-infected patients reporting truncal enlargement quantified by whole-body magnetic resonance imaging (Engelson et al., *Am. J. Clin. Nutr.,* 69(6 :1162–9 (1999)). Valk et al. used whole-body positron emission tomography (PET) imaging with [^{18}F]fluorodeoxyglucose in management of recurrent colorectal cancer (Valk et al., Arch. Surg., 134(5):503–11 (1999)). Saunders et al. evaluated fluorine-18-fluorodeoxyglucose whole body positron emission tomography imaging in the staging of lung cancer (Saunders et al., *Ann. Thorac. Surg.,* 67(3):790–7 (1999)).

U.S. Pat. No. 5,650,135 discloses a noninvasive method for detecting the localization of an entity under study from within a mammalian subject, which method comprises: (a) administering to the subject a conjugate of the entity and a light-generating moiety or a transformed cell expressing the light-generating moiety; (b) after a period of time in which the conjugate or transformed cell can achieve localization in the subject, immobilizing the subject within the detection field of a photodetector device; (c) maintaining the subject in an immobilized condition, (d) during said maintaining, measuring photon emission from the light-generating moiety, localized in the subject, with the photodetector device until an image of photon emission can be constructed; and (e) detecting said image through an opaque tissue of said mammal. U.S. Pat. No. 5,650,135 also discloses a noninvasive method for detecting the level of an entity under study in a mammalian subject over time, which method comprises: (a) administering to the subject a conjugate of the entity and a light-generating moiety or a transformed cell expressing the light-generating moiety; (b) placing the subject within the detection field of a photodetector device; (c) maintaining the subject in the detection field of the device; (d) during said maintaining, measuring photon emission from the light-generating moiety, in the subject, with the photodetector device; and (e) repeating steps (b) through (d) at selected intervals, wherein said repeating is effective to detect changes in the level of the entity in the subject over time.

Recently, Yang et al. conducted whole-body optical imaging of green fluorescent protein-expressing tumors and metastases (Yang et al., *Proc. Natl. Acad. Sci.* (*USA*), 97(3): 1206–11 (2000)). Yang et al. have imaged, in real time, fluorescent tumors growing and metastasizing in live mice. The whole-body optical imaging system is external and noninvasive. It affords unprecedented continuous visual monitoring of malignant growth and spread within intact animals. Yang et al. have established new human and rodent tumors that stably express very high levels of the Aequorea victoria green fluorescent protein (GFP) and transplanted these to appropriate animals. B16F0-GFP mouse melanoma cells were injected into the tail vein or portal vein of 6-week-old C57BL/6 and nude mice. Whole-body optical images showed metastatic lesions in the brain, liver, and bone of B 16F0-GFP that were used for real time, quantitative measurement of tumor growth in each of these organs. The AC3488-GFP human colon cancer was surgically implanted orthotopically into nude mice. Whole-body optical images showed, in real time, growth of the primary colon tumor and its metastatic lesions in the liver and skeleton. Imaging was with either a trans-illuminated epifluorescence microscope or a fluorescence light box and thermoelectrically cooled color charge-coupled device camera. The depth to which metastasis and micrometastasis could be imaged depended on their size. A 60-micrometer diameter tumor was detectable at a depth of 0.5 mm whereas a 1, 800-micrometer tumor could be visualized at 2.2-mm depth. The simple, noninvasive, and highly selective imaging of growing tumors, made possible by strong GFP fluorescence, enables the detailed imaging of tumor growth and metastasis formation. This should facilitate studies of modulators of cancer growth including inhibition by potential chemotherapeutic agents.

Methods for monitoring gene expression are known in the art (see generally, Ausubel et al. (Ed.), Current Protocols in Molecular Biology, John Wiley & Sons, Inc.). However, whole-body external optical imaging of gene expression, which offers simple, noninvasive, highly selective, and real-time recording and analysis of gene expression in an intact multi-cellular organisms, e.g., animals, is not available currently. The present invention addresses this and other related needs in the art.

DISCLOSURE OF THE INVENTION

The invention provides for whole-body external optical imaging of gene expression and methods for evaluating a candidate protocol or drug for treating diseases or disorders. The method uses a fluorophore operatively linked to the promoter of a gene and external optical imaging. Methods to screen for substances or genes that regulate target promoters are also provided.

In a specific embodiment, a method to monitor the expression of a gene is provided, which method comprises: a) delivering to a multi-cellular organism a nucleic acid encod-

ing a fluorophore operatively linked to the promoter of a gene whose expression is to be analyzed or delivering a cell containing said nucleic acid; and b) observing the presence, absence or intensity of the fluorescence generated by said fluorophore at various locations in said organism by whole-body external fluorescent optical imaging, whereby the expression of said gene is monitored.

In a preferred embodiment, a nucleic acid encoding a fluorophore operatively linked to the promoter of the gene is delivered directly to the organism. Also preferably, the nucleic acid encoding a fluorophore operatively linked to the promoter of the gene is in a viral vector such as a viral vector derived from adenovirus or a lentivirus.

In another preferred embodiment, a cell containing a nucleic acid encoding a fluorophore operatively linked to the promoter of the gene is delivered to the organism. More preferably, the cell is delivered to the organism via a surgical procedure such as direct implantation by surgical orthotopic implantation (SOI) at a desired site.

In still another preferred embodiment, the fluorophore operatively linked to the promoter of a gene is a humanized fluorophore. Also preferably, the fluorophore is a green fluorescent protein (GFP), a blue fluorescent protein (BFP) or a red fluorescent protein (RFP). More preferably, the GFP is the humanized hGFP-S65T.

In yet another preferred embodiment, the multi-cellular organism to be analyzed is a plant or an animal, including a transgenic animal. More preferably, the animal is a mammal. A human can also be analyzed by the present method.

In yet another preferred embodiment, the gene to be analyzed is expressed in a tissue or organ specific manner. More preferably, the gene is expressed in connective, epithelium, muscle or nerve tissue. Also more preferably, the gene is expressed in an internal animal organ such as brain, lung, liver, spleen, bone marrow, thymus, heart, lymph, blood, bone, cartilage, pancreas, kidney, gall bladder, stomach, intestine, testis, ovary, uterus, rectum, nervous system, gland, internal blood vessels, etc. Yet more preferably, the gene to be analyzed is a tumor or cancer associated gene such as an oncogene or a tumor suppressor gene.

In yet another preferred embodiment, the expression of more than one gene is monitored simultaneously.

In another specific embodiment, a method to evaluate a candidate protocol or drug for treating a disease or disorder is provided, which method comprises: a) administering said protocol or drug to a non-human mammalian subject which expresses a fluorophore under the direction of a promoter of a gene associated with a disease or disorder, and determining the expression of said promoter via observing the presence, absence or intensity of the fluorescence generated by said fluorophore at various locations in said mammalian subject by whole-body external fluorescent optical imaging; b) determining the expression of said promoter, via observing the presence, absence or intensity of the fluorescence generated by said fluorophore at various locations in said mammalian subject by whole-body external fluorescent optical imaging, in a control non-human mammalian subject which expresses said fluorophore under the direction of said promoter of said gene; and c) comparing the expression of said promoter determined in steps a) and b), wherein the expression determined in step a) is different from that in step b) identifies said protocol or drug as effective in treating the disease or disorder.

If overexpression of the gene is associated with the disease or disorder, the expression determined in step a) is lower than that in step b) when said protocol or drug is effective in treating the disease or disorder.

If underexpression of the gene is associated with the disease or disorder, the expression determined in step a) is higher than that in step b) when said protocol or drug is effective in treating the infection.

Preferably, the disease or disorder is a cancer, an immune system disease or disorder, a metabolism disease or disorder, a muscle and bone disease or disorder, a nervous system disease or disorder, a signal disease or disorder, or a transporter disease or disorder.

Preferably, the non-human mammalian subject which expresses a fluorophore under the direction of a promoter of the gene is produced by delivering a nucleic acid encoding the fluorophore operatively linked to the promoter of the gene, or a cell containing the nucleic acid, to the non-human mammalian subject. Alternatively, the non-human mammalian subject used in the screen is a transgenic animal.

The non-human mammalian subject used in the screening is preferably a well established laboratory animal such as a mice, a rabbit or a non-human primate.

The fluorophore used in the screening is preferably a green fluorescent protein (GFP), a blue fluorescent protein (BFP) or a red fluorescent protein (RFP).

More than one candidate protocol or candidate drug is preferably screened for simultaneously.

If the non-human mammalian subject expresses a fluorophore under the direction of a promoter of an infectious organism, the expression determined in step a) is lower than that in step b) when said protocol or drug is effective in treating infection caused by the infectious organism.

The non-human mammalian subject used in the screening is preferably an infectious disease animal model.

The infectious organism screened against is preferably a fungus such as a yeast, a bacterium such as an eubacteria or an archaebacteria, or a virus such as a Class I virus, a Class II virus, a Class III virus, a Class IV virus, a Class V virus or a Class VI virus.

If the infection is caused by a bacterium, the candidate drug to be screened is preferably an antibiotic.

In still another specific embodiment, a method to screen for a modulator of the expression of a gene in a non-human multi-cellular organism is provided, which method comprises: a) administering a test substance to a non-human multi-cellular organism which expresses a fluorophore under the direction of a promoter of a gene, and determining the expression of said promoter via observing the presence, absence or intensity of the fluorescence generated by said fluorophore at various locations in said multi-cellular organism by whole-body external fluorescent optical imaging; b) determining the expression of said promoter, via observing the presence, absence or intensity of the fluorescence generated by said fluorophore at various locations by whole-body external fluorescent optical imaging, in a control multi-cellular organism which expresses said fluorophore under the direction of said promoter of said gene; and c) comparing the expression of said promoter determined in steps a) and b), wherein the expression determined in step a) is different from that in step b) identifies said test substance as a modulator of said gene expression. Preferably, the promoter is an endogenous promoter of the multi-cellular organism.

In yet another specific embodiment, a method to screen for a non-human multi-cellular organism that expresses a gene at an altered level is provided, which method com-

prises: a) administering a mutation-inducing agent or treatment to a non-human multi-cellular organism which expresses a fluorophore under the direction of a promoter of a gene, and determining the expression of said promoter via observing the presence, absence or intensity of the fluorescence generated by said fluorophore at various locations in said multi-cellular organism by whole-body external fluorescent optical imaging; b) determining the expression of said promoter, via observing the presence, absence or intensity of the fluorescence generated by said fluorophore at various locations by whole-body external fluorescent optical imaging in an untreated control multi-cellular organism which expresses said fluorophore under the direction of said promoter of said gene; and c) comparing the expression of said promoter determined in steps a) and b), wherein the expression determined in step a) is different from that in step b) identifies a multi-cellular organism that expresses said gene at the altered level. Preferably, the mutation-inducing agent or treatment causes a mutation in germ-line cells of the multi-cellular organism so that the desired mutation is stably-transferable to offspring of the multi-cellular organism.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. **1**A and **1**B show the time course of expression of adenoviral-administered GFP in brain and liver respectively. Fluorescence first becomes visible in the brain within six (6) hours after local delivery and liver fluorescence became detectable at about seven (7) hours after injection into the tail vein.

FIGS. **2**A and **2**B art pertinent to administration of lentiviral vectors. FIG. **2**A is a diagram of lentiviral vector GFP-LV. FIG. **2**B is a diagram of a control observation method; whole body measurement involved use of a light box.

MODES FOR CARRYING OUT THE INVENTION

A. Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of ordinary skill in the art to which this invention belongs. All patents, applications, published applications and other publications and sequences from GenBank and other data bases referred to herein are incorporated by reference in their entirety.

As used herein, "delivering a nucleic acid to a multi-cellular organism" refers to a process in which the nucleic acid is either administered directly into the body of the multi-cellular organism, or the nucleic acid is administered into a cell first, and then the cell containing the nucleic acid is administered into the body of the multi-cellular organism. After delivery into the organism, the nucleic acid may exist independently from the genome of the host organism or may be integrated into the genome of the host organism. If the nucleic acid is integrated into a germline cell of the host organism, such nucleic acid may be transmitted into the host organism's offspring.

As used herein, "whole-body external fluorescent optical imaging" refers to an imaging process in which the presence, absence or intensity of the fluorescence generated by the fluorophore at various locations in the host organism is monitored, recorded and/or analyzed externally without any procedure, e.g., surgical procedure, to expose and/or to excise the desired observing site from the host organism. To achieve the whole-body external fluorescent optical imaging, it is necessary that the intensity of the fluorescence generated by the fluorophore is sufficiently high so that, even when the fluorescence site is an internal one within the host organism body, the fluorescence signal can be analyzed externally without exposing or excising the site from the host body, or while the animal is not controlled.

As no invasive procedures are required and the intensity of the signal is sufficiently great for direct observation, the animal may remain completely mobile and need not be restrained. The ability to provide a completely non-invasive observation protocol is highly significant. If the animal is traumatized either by, e.g., incision or by physical restraint, e.g., straps or pins, the alteration in metabolism may affect the expression of the genes in organs or tissues.

Since whole-body external fluorescent optical imaging are quick and easily amenable to automation, it can be used for monitoring large number of gene expression simultaneously. In addition, it can be employed in high-throughput screening methods for identifying protocols, substances including candidate drugs, and cis-acting regulators that regulate the expression of a target gene. Using the whole-body external fluorescent optical imaging provided in this application, multiple candidate protocols, substances, drugs, and cis-acting regulators can be screened for, either against a single target gene or against multiple target genes, in either a single animal or in multiple animals, simultaneously.

As used herein, "fluorophore" refers to a protein that is auto-fluorescent such that no other substrates or co-factors are needed for it to fluoresce. Non-limiting examples of such fluorophores include green fluorescent proteins (GFPs), blue fluorescent proteins (BFPs) and red fluorescent protein (RFPs), and functional fragments, derivatives and analogues thereof.

As used herein, "a promoter region or promoter element" refers to a segment of DNA or RNA that controls transcription of the DNA or RNA to which it is operatively linked. The promoter region includes specific sequences that are sufficient for RNA polymerase recognition, binding and transcription initiation. This portion of the promoter region is referred to as the promoter. In addition, the promoter region includes sequences that modulate this recognition, binding and transcription initiation activity of RNA polymerase. These sequences may be cis acting or may be responsive to trans acting factors. Promoters, depending upon the nature of the regulation, may be constitutive or regulated.

As used herein, "operatively linked or operationally associated" refers to the functional relationship of DNA with regulatory and effector sequences of nucleotides, such as promoters, enhancers, transcriptional and translational stop sites, and other signal sequences. For example, operative linkage of DNA to a promoter refers to the physical and functional relationship between the DNA and the promoter such that the transcription of such DNA is initiated from the promoter by an RNA polymerase that specifically recognizes, binds to and transcribes the DNA. In order to optimize expression and/or in vitro transcription, it may be necessary to remove, add or alter 5' untranslated portions of the clones to eliminate extra, potential inappropriate alternative translation initiation (i.e., start) codons or other sequences that may interfere with or reduce expression, either at the level of transcription or translation. Alternatively, consensus ribosome binding sites (see, e.g., Kozak, *J. Biol. Chem.,* 266:19867–19870 (1991)) can be inserted immediately 5' of the start codon and may enhance expression. The desirability of (or need for) such modification may be empirically determined.

As used herein, "humanized fluorophore" refers to a fluorophore whose codon is modified according to the codon usage pattern in human genome to enhance its expression while substantially maintaining its fluorescent characteristics.

As used herein, "multi-cellular organism" refers to an organism with certain cell numbers, mass, and internal structure so that internal sites of such multi-cellular organism are not externally detectable by non-fluorescent optical imaging without exposing the internal sites. Sufficiently high intensity of internal fluorescence is needed for external fluorescent optical imaging of the internal site.

As used herein, "plant" refers to any of various photosynthetic, eucaryotic multi-cellular organisms of the kingdom Plantae, characteristically producing embryos, containing chloroplasts, having cellulose cell walls and lacking locomotion.

As used herein, "animal" refers to a multi-cellular organism of the kingdom of Animalia, characterized by a capacity for locomotion, nonphotosynthetic metabolism, pronounced response to stimuli, restricted growth and fixed bodily structure. Non-limiting examples of animals include birds such as chickens, vertebrates such fish and mammals such as mice, rats, rabbits, cats, dogs, pigs, cows, ox, sheep, goats, horses, monkeys and other non-human primates.

As used herein, "expressed in a tissue or organ specific manner" refers to a gene expression pattern in which a gene is expressed, either transiently or constitutively, only in certain tissues or organs, but not in other tissues or organs.

As used herein, "tissue" refers to a collection of similar cells and the intracellular substances surrounding them. There are four basic tissues in the body: 1) epithelium; 2) connective tissues, including blood, bone, and cartilage; 3) muscle tissue; and 4) nerve tissue.

As used herein, "organ" refers to any part of the body exercising a specific function, as of respiration, secretion or digestion.

As used herein, "disease or disorder" refers to a pathological condition in an organism resulting from, e.g., infection or genetic defect, and characterized by identifiable symptoms.

As used herein, neoplasm (neoplasia) refers to abnormal new growth, and thus means the same as tumor, which may be benign or malignant. Unlike hyperplasia, neoplastic proliferation persists even in the absence of the original stimulus.

As used herein, cancer refers to a general term for diseases caused by any type of malignant tumor.

As used herein, "oncogene" refers to a mutated and/or overexpressed version of a normal gene of animal cells (the proto-oncogene) that in a dominant fashion can release the cell from normal restraints on growth, and thus alone, or in concert with other changes, convert a cell into a tumor cell. Exemplary oncogenes include, but are not limited to, abl, erbA, erbB, ets, fes (fps), fgr, fms, fos, hst, int1, int2, jun, hit, B-lym, mas, met, mil (raf), mos, myb, myc, N-myc, neu (ErbB2), ral (mil), Ha-ras, Ki-ras, N-ras, rel, ros, sis, src, ski, trk and yes.

As used herein, "tumor suppressor gene" (or anti-oncogene, cancer susceptibility gene) refers to a gene that encodes a product which normally negatively regulates the cell cycle, and which must be mutated or otherwise inactivated before a cell can proceed to rapid division. Exemplary tumor suppressor genes include, but are not limited to, p16, p21, p53, RB (retinoblastoma), WT-1 (Wiln's tumor), DCC (deleted in colonic carcinoma), NF-1 (neurofibrosarcoma) and APC (adenomatous polypospis coli).

As used herein, "an immune system disease or disorder" refers to a pathological condition caused by a defect in the immune system. The immune system is a complex and highly developed system, yet its mission is simple: to seek and kill invaders. If a person is born with a severely defective immune system, death from infection by a virus, bacterium, fungus or parasite will occur. In severe combined immunodeficiency, lack of an enzyme means that toxic waste builds up inside immune system cells, killing them and thus devastating the immune system. A lack of immune system cells is also the basis for DiGeorge syndrome: improper development of the thymus gland means that T cell production is diminished. Most other immune disorders result from either an excessive immune response or an 'autoimmune attack'. For example, asthma, familial Mediterranean fever and Crohn disease (inflammatory bowel disease) all result from an over-reaction of the immune system, while autoimmune polyglandular syndrome and some facets of diabetes are due to the immune system attacking 'self' cells and molecules. A key part of the immune system's role is to differentiate between invaders and the body's own cells—when it fails to make this distinction, a reaction against 'self' cells and molecules causes autoimmune disease.

As used herein, "a metabolism disease or disorder" refers to a pathological condition caused by errors in metabolic processes. Metabolism is the means by which the body derives energy and synthesizes the other molecules it needs from the fats, carbohydrates and proteins we eat as food, by enzymatic reactions helped by minerals and vitamins. There is a significant level of tolerance of errors in the system: often, a mutation in one enzyme does not mean that the individual will suffer from a disease. A number of different enzymes may compete to modify the same molecule, and there may be more than one way to achieve the same end result for a variety of metabolic intermediates. Disease will only occur if a critical enzyme is disabled, or if a control mechanism for a metabolic pathway is affected.

As used herein, "a muscle and bone disease or disorder" refers to a pathological condition caused by defects in genes important for the formation and function of muscles, and connective tissues. Connective tissue is used herein as a broad term that includes bones, cartilage and tendons. For example, defects in fibrillin—a connective tissue protein that is important in making the tissue strong yet flexible—cause Marfan syndrome, while diastrophic dysplasia is caused by a defect in a sulfate transporter found in cartilage. Two diseases that originate through a defect in the muscle cells themselves are Duchenne muscular dystrophy (DMD) and myotonic dystrophy (DM). DM is another 'dynamic mutation' disease, similar to Huntington disease, that involves the expansion of a nucleotide repeat, this time in a muscle protein kinase gene. DMD involves a defect in the cytoskeletal protein, dystrophin, which is important for maintaining cell structure.

As used herein, "a nervous system disease or disorder" refers to a pathological condition caused by defects in the nervous system including the central nervous system, i.e., brain, and the peripheral nervous system. The brain and nervous system form an intricate network of electrical signals that are responsible for coordinating muscles, the senses, speech, memories, thought and emotion. Several diseases that directly affect the nervous system have a genetic component: some are due to a mutation in a single gene, others are proving to have a more complex mode of

inheritance. As our understanding of the pathogenesis of neurodegenerative disorders deepens, common themes begin to emerge: Alzheimer brain plaques and the inclusion bodies found in Parkinson disease contain at least one common component, while Huntington disease, fragile X syndrome and spinocerebellar atrophy are all 'dynamic mutation' diseases in which there is an expansion of a DNA repeat sequence. Apoptosis is emerging as one of the molecular mechanisms invoked in several neurodegenerative diseases, as are other, specific, intracellular signaling events. The biosynthesis of myelin and the regulation of cholesterol traffic also figure in Charcot-Marie-Tooth and Neimann-Pick disease, respectively.

As used herein, "a signal disease or disorder" refers to a pathological condition caused by defects in the signal transduction process. Signal transduction within and between cells mean that they can communicate important information and act upon it. Hormones released from their site of synthesis carry a message to their target site, as in the case of leptin, which is released from adipose tissue (fat cells) and transported via the blood to the brain. Here, the leptin signals that enough has been eaten. Leptin binds to a receptor on the surface of hypothalamus cells, triggering subsequent intracellular signaling networks. Intracellular signaling defects account for several diseases, including cancers, ataxia telangiectasia and Cockayne syndrome. Faulty DNA repair mechanisms are also invoked in pathogenesis, since control of cell division, DNA synthesis and DNA repair all are inextricably linked. The end-result of many cell signals is to alter the expression of genes (transcription) by acting on DNA-binding proteins. Some diseases are the result of a lack of or a mutation in these proteins, which stop them from binding DNA in the normal way. Since signaling networks impinge on so many aspects of normal function, it is not surprising that so many diseases have at least some basis in a signaling defect.

As used herein, "a transporter disease or disorder" refers to a pathological condition caused by defects in a transporter, channel or pump. Transporters, channels or pumps that reside in cell membranes are key to maintaining the right balance of ions in cells, and are vital for transmitting signals from nerves to tissues. The consequences of defects in ion channels and transporters are diverse, depending on where they are located and what their cargo is. For example, in the heart, defects in potassium channels do not allow proper transmission of electrical impulses, resulting in the arrhythmia seen in long QT syndrome. In the lungs, failure of a sodium and chloride transporter found in epithelial cells leads to the congestion of cystic fibrosis, while one of the most common inherited forms of deafness, Pendred syndrome, looks to be associated with a defect in a sulphate transporter.

As used herein, "infection" refers to invasion of the body of a multi-cellular organism with organisms that have the potential to cause disease.

As used herein, "infectious organism" refers to an organism that is capable to cause infection of a multi-cellular organism. Most infectious organisms are microorganisms such as viruses, bacteria and fungi.

As used herein, "bacteria" refers to small prokaryotic organisms (linear dimensions of around 1 μm) with non-compartmentalized circular DNA and ribosomes of about 70S. Bacteria protein synthesis differs from that of eukaryotes. Many anti-bacterial antibiotics interfere with bacteria proteins synthesis but do not affect the infected host.

As used herein, "eubacteria" refers to a major subdivision of the bacteria except the archaebacteria. Most Gram-positive bacteria, cyanobacteria, mycoplasmas, enterobacteria, pseudomonas and chloroplasts are eubacteria. The cytoplasmic membrane of eubacteria contains ester-linked lipids; there is peptidoglycan in the cell wall (if present); and no introns have been discovered in eubacteria.

As used herein, "archaebacteria" refers to a major subdivision of the bacteria except the eubacteria. There are 3 main orders of archaebacteria: extreme halophiles, methanogens and sulphur-dependent extreme thermophiles. Archaebacteria differs from eubacteria in ribosomal structure, the possession (in some case) of introns, and other features including membrane composition.

As used herein, "virus" refers to obligate intracellular parasites of living but non-cellular nature, consisting of DNA or RNA and a protein coat. Viruses range in diameter from about 20 to about 300 nm. Class I viruses (Baltimore classification) have a double-stranded DNA as their genome; Class II viruses have a single-stranded DNA as their genome; Class III viruses have a double-stranded RNA as their genome; Class IV viruses have a positive single-stranded RNA as their genome, the genome itself acting as mRNA; Class V viruses have a negative single-stranded RNA as their genome used as a template for mRNA synthesis; and Class VI viruses have a positive single-stranded RNA genome but with a DNA intermediate not only in replication but also in mRNA synthesis. The majority of viruses are recognized by the diseases they cause in plants, animals and prokaryotes. Viruses of prokaryotes are known as bacteriophages.

As used herein, "fungi" refers to a division of eucaryotic organisms that grow in irregular masses, without roots, stems, or leaves, and are devoid of chlorophyll or other pigments capable of photosynthesis. Each organism (thallus) is unicellular to filamentous, and possess branched somatic structures (hyphae) surrounded by cell walls containing glucan or chitin or both, and containing true nuclei.

As used herein, "antibiotic" refers to a substance either derived from a mold or bacterium or organically synthesized, that inhibits the growth of certain microorganisms without substantially harming the host of the microorganisms to be killed or inhibited.

As used herein, "test substance" refers to a chemically defined compound (e.g., organic molecules, inorganic molecules, organic/inorganic molecules, proteins, peptides, nucleic acids, oligonucleotides, lipids, polysaccharides, saccharides, or hybrids among these molecules such as glycoproteins, etc.) or mixtures of compounds (e.g., a library of test compounds, natural extracts or culture supernatants, etc.) whose effect on the promoter to be analyzed is determined by the disclosed and/or claimed methods herein.

For clarity of disclosure, and not by way of limitation, the detailed description of the invention is divided into the subsections that follow.

B. Methods of Whole-Body External Optical Imaging of Gene Expression

In a specific embodiment, a method to monitor the expression of a gene is provided herein, which method comprises: a) delivering to a multi-cellular organism a nucleic acid encoding a fluorophore operatively linked to the promoter of a gene whose expression is to be analyzed or a cell containing said nucleic acid; and b) observing the presence, absence or intensity of the fluorescence generated by said fluorophore at various locations in said organism by whole-body external fluorescent optical imaging, whereby the expression of said gene is monitored.

The present methods can be used to monitor gene expression for any suitable purposes including prognostic, diag-

nostic and screening purposes. For example, if abnormal gene expression is associated with a disease or disorder in a multi-cellular organism such as a plant or an animal, the present method can be used in prognosis or diagnosis by monitoring the abnormal gene expression. The present monitoring methods are advantageous over the currently available gene expression monitoring methods in several aspects. First, the present monitoring methods can avoid any invasive procedures and this is particularly advantageous for human clinical uses. Second, the present monitoring methods offer in vivo, real-time and continuous monitor and analysis of gene expression in plants or animals, which cannot be accomplished using the currently available monitoring methods. Third, the present monitoring methods are quick and easily amenable to automation, which are important for monitoring large number of gene expression simultaneously. Since many diseases or disorders involve abnormal gene expression of more than gene, the present monitoring methods are particularly suitable for the prognosis and diagnosis of these diseases or disorders. Besides prognosis or diagnosis, if expression of certain genes is a good indicator of tissue or organ health or functionality, the present monitoring methods can also be used in monitoring the health or functionality of these tissues or organs without any invasive procedures.

1. Methods for Delivering the Nucleic Acids into the Multi-Cellular Organism

The nucleic acids encoding a fluorophore operatively linked to the promoter of a gene whose expression is to be analyzed can be a DNA or a RNA. Such nucleic acids can be delivered into the body of the multi-cellular organism by any methods known in the art.

For example, if the host multi-cellular organism is an animal, the DNA or RNA sequence can be delivered to the interstitial space of tissues of the animal body, including those of muscle, skin, brain, lung, liver, spleen, bone marrow, thymus, heart, lymph, blood, bone, cartilage, pancreas, kidney, gall bladder, stomach, intestine, testis, ovary, uterus, rectum, nervous system, eye, gland, and connective tissue. Interstitial space of the tissues comprises the intercellular, fluid, mucopolysaccharide matrix among the reticular fibers or organ tissues, elastic fibers in the walls of vessels or chambers, collagen fibers of fibrous tissues, or that same matrix within connective tissue ensheathing muscle cells or in the lacunae of bone. It is similarly the space occupied by the plasma of the circulation of the lymph fluid of the lymphatic channels.

The DNA or RNA sequence can be conveniently delivered by injection into the tissues comprising these cells. They are preferably delivered to and expressed in persistent, non-dividing cells which are differentiated, although delivery and expression can be achieved in non-differentiated or less completely differentiated cells, such as, for example, stem cells of blood or skin fibroblasts.

In a specific embodiment, the DNA or RNA sequence is delivered directly to a tissue of the host animal. Preferably, the DNA or RNA sequence is delivered directly to muscle, skin or mucous membrane. Delivery to the interstitial space of muscle tissue is preferred because muscle cells are particularly competent in their ability to take up and express polynucleotides.

The DNA or RNA sequence can be delivered directly to a tissue of the host animal by injection, by gene gun technology or by lipid mediated delivery technology. The injection can be conducted via a needle or other injection devices. The gene gun technology is disclosed in U.S. Pat. No. 5,302,509 and the lipid mediated delivery technology is disclosed in U.S. Pat. No. 5,703,055.

In still another specific embodiment, the DNA or RNA sequence is delivered to a cell of host animal and said cell containing the DNA or RNA sequence is delivered to a suitable tissue of the host animal. Preferably, the DNA or RNA sequence is delivered to tail or portal vein of the host animal.

The DNA or RNA sequence can be delivered to the cells of the host animal by a number of methods (see generally Koprowski & Weiner, DNA vaccination/genetic vaccination, 1998. Springer-verlag Berlin Heidelberg) including $Ca_3(PO_4)_2$-DNA transfection (Sambrook et al., *Molecular Cloning,* 2nd Edition, Plainview, N.Y. Cold Spring Harbor Press, 1989), DEAE dextran-DNA transfection (Sambrook et al., *Molecular Cloning,* 2nd Edition, Plainview, N.Y. Cold Spring Harbor Press, 1989), electroporation (e.g., protocols from Bio-Rad), transfection using "LIPOFECTIN"™ reagent (e.g., protocols from BRL-Life Science), gene gun technology (U.S. Pat. No. 5,302,509), or viral gene delivery system (Kaplitt et al., *Viral Vectors, Academic Press, Inc.,* 1995).

Gold-particle based gene gun delivery is disclosed in U.S. Pat. No. 5,302,509. In a specific embodiment, Bio-Rad helios gene gun system is used in the DNA delivery. (BIO-RAD Inc. New England). The helios gene gun is a convenient, hand-held device that provides rapid and direct gene transfer in vivo. The device employs an adjustable, helium pulse to sweep DNA coated gold microcarriers from the inner wall of a small plastic cartridge directly into the target cells. The tubing prepstation and tubing cutter provide a simple way to prepare 50 cartridge "bullets" at a time.

In a preferred embodiment, a nucleic acid encoding a fluorophore operatively linked to the promoter of the gene is delivered directly to the organism. More preferably, the nucleic acid encoding a fluorophore operatively linked to the promoter of the gene is delivered to the organism, or to a cell to be delivered to the organism, in a viral vector such as a viral vector derived from adenovirus or a lentivirus.

Any viral vectors known in the art can be used. For example, vectors derived from a parvovirus (U.S. Pat. Nos. 5,252,479 and 5,624,820), a paramyxovirus such as simian virus 5 (SV5) (U.S. Pat. No. 5,962,274), a retrovirus such as HIV (U.S. Pat. Nos. 5,753,499 and 5,888,767), and a baculovirus such as a nuclear polyhedrosis virus (U.S. Pat. No. 5,674,747) can be used. Preferably, a vector derived from adenovirus can be used (U.S. Pat. Nos. 5,670,488, 5,817,492, 5,820,868, 5,856,152, 5,981,225).

U.S. Pat. No. 5,670,488 discloses an adenoviral vector comprising an adenovirus genome from which one or more of the E4 open reading frames has been deleted, but retaining sufficient E4 sequences to promote virus replication in vitro, and additionally comprising a DNA sequence of interest operably linked to expression control sequences and inserted into said adenoviral genome.

U.S. Pat. No. 5,817,492 discloses a recombinant adenoviral vector comprising: two DNA sequences which serve as a substrate for a recombinase enzyme, an origin of replication which is operable in an animal cell, a promoter, a foreign gene and a poly(A) sequence, wherein said origin of replication, promoter, foreign gene and poly(A) sequence are located between the two DNA sequences, and wherein said vector contains an E1A gene region deletion.

U.S. Pat. No. 5,820,868 discloses a live recombinant bovine adenovirus vector (BAV) wherein a part or all of the E3 multiple gene coding region is replaced by a heterolo-

gous nucleotide sequence encoding a foreign gene or fragment thereof. It also discloses a live recombinant bovine adenovirus vector (BAV) wherein part or all of the E3 multiple gene coding region is replaced by a heterologous nucleotide sequence encoding a foreign gene or fragment thereof and wherein said heterologous nucleotide sequence is optionally under the control of a promoter not normally associated with either said foreign gene or the bovine adenovirus genome.

U.S. Pat. No. 5,856,152 discloses a hybrid viral vector comprising: (a) adenovirus sequences comprising the adenovirus 5'and 3' cis-elements necessary for replication and virion encapsidation; and (b) adeno-associated virus sequences comprising the 5' and 3' ITRs of an adeno-associated virus, said adeno-associated virus sequences flanked by the adenoviral sequences of (a); and (c) a selected gene operatively linked to regulatory sequences which direct its expression in a target cell, said gene and regulatory sequences flanked by the adeno-associated virus sequences of (b).

U.S. Pat. No. 5,981,225 discloses a gene transfer vector consisting essentially of, in 5' to 3' orientation, the following elements: (i) a first adenovirus inverted terminal repeat, (ii) an adenoviral VAI gene and/or VAII gene, (iii) a gene foreign to adenovirus, wherein said gene is operably linked to a promoter functional in adenovirus target cells, and (iv) a second adenovirus inverted terminal repeat, wherein the order of elements (ii) and (iii) may be reversed; and wherein one or both of element (i) and element (iv) additionally comprise an adenovirus packaging signal, and wherein said vector is incapable of producing, in vitro, recombinant adenovirus virus particles which have encapsidated therein said vector unless said vector is co-transfected or co-infected into adenovirus host cells with adenovirus genomic DNA or adenovirus particles containing adenovirus genomic DNA, respectively.

In another preferred embodiment, cells containing a nucleic acid encoding a fluorophore operatively linked to the promoter of the gene are delivered to the organism. More preferably, the cells are delivered to the organism via a surgical procedure such as direct implantation by surgical orthotopic implantation (SOI) at a desired site (see e.g., Chang et al., *Anticancer Res.,* 19(5B):4199 (1999); and An et al., *Prostate,* 34(3 :169–74 (1998)).

It will be understood, that by introducing a nucleic acid molecule wherein a promoter is coupled to a nucleotide sequence encoding a fluorescent reporter gene, the introduced nucleic acid molecule can be used as a surrogate for the endogenous promoter. Thus, if the endogenous gene is over-expressed or under-expressed in the context of a particular condition, the behavior of the introduced construct will mimic that of the endogenous promoter. It is not necessary that the reporter-encoding nucleotide sequence be operably linked only to a promoter; the nucleotide sequence encoding reporter may be introduced into the nucleotide sequence encoding the protein normally under control of the promoter or coupled to another protein. Any method of operably linking the nucleotide sequence encoding reporter to the control sequences for the gene whose expression is to be monitored falls within the scope of the invention.

It will be seen that there are a number of ways to introduce this construct. First, the nucleic acid comprising the reporter encoding nucleotide sequence operably linked to the control sequences/promoter of interest can be introduced to the multicellular organism by direct injection, but preferably using a viral vector, such as a adenoviral vector or a antiviral vector. Since the introduced construct is not endogenous, the expression of this construct essentially functions as a surrogate for the endogenous gene. That is, the same influences which influence the endogenous gene will also influence the introduced construct. Thus, the conclusions reached by observing the expression of the construct, including the effects of various treatments on such expression, can be extrapolated to, and are equally valid for, the counterpart endogenous gene.

Second, the reporter encoding nucleotide sequence could be introduced into the cells of a particular tissue by targeting to the promoter to be studied and inserted using position-specific techniques, such as homologous recombination. When this method is used, the expression of the endogenous promoter can be observed directly as well as can the effect of various treatments thereon.

Third, a construct such as those described for the first method can be provided to embryonic tissue to obtain transgenic organisms where the reporter construct is itself endogenous, see, for example, Fukumura, D., et al., *Cell* (1998) 94:715–725, incorporated herein by reference, which describes transgenic mice which use GFP as a reporter for VEGF promoter activity.

Techniques for all three methods are well known in the art.

2. Fluorophores

Any fluorophores known in the art can be used in the present methods. In a preferred embodiment, the fluorophore operatively linked to the promoter of a gene is a humanized fluorophore. Also preferably, the fluorophore is a green fluorescent protein (GFP), a blue fluorescent protein (BFP) and a red fluorescent protein (RFP). More preferably, the GFP is the humanized hGFP-S65T.

The native gene encoding GFP has been cloned from the bioluminescent jellyfish Aequorea victoria (Morin et al., *J. Cell Physiol.,* 77:313–318 (1972)). The availability of the gene has made it possible to use GFP as a marker for gene expression. GFP itself is a 283 amino acid protein with a molecular weight of 27 kD. It requires no additional proteins from its native source nor does it require substrates or cofactors available only in its native source in order to fluoresce (Prasher et al., *Gene,* 111:229–233 (1992); Yang et al., *Nature Biotechnol.,* 14:1252–1256 (1996); and Cody et al., *Biochemistry,* 32:1212–1218 (1993)). Mutants of the GFP gene have been found useful to enhance expression and to modify excitation and fluorescence. GFP-S65T (wherein serine at 65 is replaced with threonine) is particularly useful in the invention method and has a single excitation peak at 490 nm. (Heim et al., *Nature,* 373:663–664 (1995)); and U.S. Pat. No. 5,625,048). Other mutants have also been disclosed by Delagrade et al., *Biotechnology,* 13:151–154 (1995); Cormack et al., *Gene,* 173:33–38 (1996); and Cramer et al. *Nature Biotechnol.,* 14:315–319 (1996). Additional mutants are also disclosed in U.S. Pat. No. 5,625,048. By suitable modification, the spectrum of light emitted by the GFP can be altered. Thus, although the term "GFP" is used in the present application, the proteins included within this definition are not necessarily green in appearance. Various forms of GFP exhibit colors other than green and these, too, are included within the definition of "GFP" and are useful in the methods and materials of the invention. In addition, it is noted that green fluorescent proteins falling within the definition of "GFP" herein have been isolated from other organisms, such as the sea pansy, *Renilla reriformis*. Any suitable and convenient form of the GFP gene can be used in the invention. Techniques for labeling cells in general using GFP are disclosed in U.S. Pat. No. 5,491,084 (supra).

Other GFP, BFP and RFP can be used in the present methods. For instances, the green fluorescent proteins encoded by nucleic acids with the following GenBank accession Nos. can be used: U47949 (AGP1); U43284; AF007834 (GFPuv); U89686 (*Saccharomyces cerevisiae* synthetic green fluorescent protein (cox3::GFPm-3) gene); U89685 (*Saccharomyces cerevisiae* synthetic green fluorescent protein (cox3::GFPm) gene); U87974 (Synthetic construct modified green fluorescent protein GFP5-ER (mgfp5-ER)); U87973 (Synthetic construct modified green fluorescent protein GFP5 (mgfp5)); U87625 (Synthetic construct modified green fluorescent protein GFP-ER (mfgp4-ER)); U87624 (Synthetic construct green fluorescent protein (mgfp4) mRNA)); U73901 (Aequorea victoria mutant 3); U50963 (Synthetic); U70495 (soluble-modified green fluorescent protein (smGFP)); U57609 (enhanced green fluorescent protein gene); U57608 (enhanced green fluorescent protein gene); U57607 (enhanced green fluorescent protein gene); U57606 (enhanced green fluorescent protein gene); U55763 (enhanced green fluorescent protein (egfp); U55762 (enhanced green fluorescent protein (egfp); U55761 (enhanced green fluorescent protein (egfp); U54830 (Synthetic *E. coli* Tn3-derived transposon green fluorescent protein (GF); U36202; U36201; U19282; U19279; U19277; U19276; U19281; U19280; U19278; L29345 (Aequorea victoria); M62654 (Aequorea victoria); M62653 (Aequorea victoria); AAB47853 ((U87625) synthetic construct modified green fluorescent protein (GFP-ER)); AAB47852 ((U87624) synthetic construct green fluorescent protein).

Similarly, the blue fluorescent proteins encoded by nucleic acids with the following GenBank accession Nos. can be used: U70497 (soluble-modified blue fluorescent protein (smBFP); 1BFP (blue variant of green fluorescent protein); AAB16959 (soluble-modified blue fluorescent protein).

Also similarly, the red fluorescent proteins encoded by nucleic acids with the following GenBank accession Nos. can be used: U70496 (soluble-modified red-shifted green fluorescent protein (smRSGFP); AAB16958 (U70496) soluble-modified red-shifted green fluorescent protein).

A fluorophore that changes color with time is reported by Teiskikh, A., et al., *Science* (2000) 290:1585–1588, incorporated herein by reference. This permits tracing time dependent expression.

3. Multi-Cellular Organisms

The present methods can be used in monitoring gene expression in any suitable multi-cellular organisms. In a preferred embodiment, the multi-cellular organism to be analyzed is a plant or an animal, including a transgenic animal. More preferably, the animal is a mammal including a human. Animals that can be analyzed with the present monitoring emthods include, but are not limited to, mice, rats, rabbits, cats, dogs, pigs, cows, ox, sheep, goats, horses, monkeys and other non-human primates.

4. Tissue or Organ Specific Gene Expression

The present methods can be used in monitoring expression of genes that are expressed in a tissue or organ specific manner. The present methods can be used in monitoring health and/or functionality of tissues and/or organs if expression pattern of certain genes are associated with health and/or functionality of these tissues and organs. Preferably, the gene to be monitored is expressed in connective, epithelium, muscle or nerve tissue. Also preferably, the gene to be monitored is expressed in an accessory organ of the eye, annulospiral organ, auditory organ, Chievitz organ, circumventricular organ, Corti organ, critical organ, enamel organ, end organ, external female gential organ, external male genital organ, floating organ, flower-spray organ of Ruffini, genital organ, Golgi tendon organ, gustatory organ, organ of hearing, internal female genital organ, internal male genital organ, intromittent organ, Jacobson organ, neurohemal organ, neurotendinous organ, olfactory organ, otolithic organ, ptotic organ, organ of Rosenmüller, sense organ, organ of smell, spiral organ, subcommissural organ, subfornical organ, supernumerary organ, tactile organ, target organ, organ of taste, organ of touch, urinary organ, vascular organ of lamina terminalis, vestibular organ, vestibulocochlear organ, vestigial organ, organ of vision, visual organ, vomeronasal organ, wandering organ, Weber organ and organ of Zuckerkandl. More preferably, the gene to be monitored is expressed in an internal animal organ such as brain, lung, liver, spleen, bone marrow, thymus, heart, lymph, blood, bone, cartilage, pancreas, kidney, gall bladder, stomach, intestine, testis, ovary, uterus, rectum, nervous system, gland, internal blood vessels, etc.

In other embodiments, the fluorophore, e.g., GFP, BFP or RFP, can be operatively linked to the following animal transcriptional control regions that exhibit tissue specificity to monitor these tissue specific gene expressions in animals: elastase I gene control region which is active in pancreatic acinar cells (Swift et al., *Cell* 38:639–646 (1984); Ornitz et al., *Cold Spring Harbor Symp. Quant. Biol.* 50:399–409 (1986); MacDonald, *Hepatology* 7:425–515 (1987)); insulin gene control region which is active in pancreatic beta cells (Hanahan et al., *Nature* 315:115–122 (1985)), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al., *Cell* 38:647–658 (1984); Adams et al., *Nature* 318:533–538 (1985); Alexander et al., *Mol. Cell Biol.* 7:1436–1444 (1987)), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al., *Cell* 45:485–495 (1986)), albumin gene control region which is active in liver (Pinckert et al., *Genes and Devel.* 1:268–276 (1987)), alpha-fetoprotein gene control region which is active in liver (Krumlauf et al., *Mol. Cell. Biol.* 5:1639–1648 (1985); Hammer et al., *Science* 235:53–58 1987)), alpha-1 antitrypsin gene control region which is active in liver (Kelsey et al., *Genes and Devel.* 1:161–171 (1987)), beta globin gene control region which is active in myeloid cells (Mogram et al., *Nature* 315:338–340 (1985); Kollias et al., *Cell* 46:89–94 (1986)), myelin basic protein gene control region which is active in oligodendrocyte cells of the brain (Readhead et al., *Cell* 48:703–712 (1987)), myosin light chain-2 gene control region which is active in skeletal muscle (Sani, *Nature* 314:283–286 (1985)), and gonadotrophic releasing hormone gene control region which is active in gonadotrophs of the hypothalamus (Mason et al., *Science* 234:1372–1378 (1986)).

5. Tumor or Cancer Associated Gene Expression

The present methods can be used in monitoring expression of genes that are specifically expressed in tumors or cancers. Preferably, the gene to be analyzed is a tumor or cancer associated gene such as an oncogene or a tumor suppressor gene. For instance, the expression of the oncogenes listed in the following Table 1 can be monitored by the present methods.

TABLE 1

Oncogenes and tumor viruses

Acronym	Virus	Species	Tumor origin	Comments
abl	Abelson leukemia	Mouse	Chronic myelogenous leukemia	TyrPK(src)
erbA	Erythroblastosis	Chicken		Homology to human glucocorticoid receptor
erbB	Erythroblastosis	Chicken		TryPK EGF/TGFc receptor
ets	E26 myeloblastosis	Chicken		Nuclear
fes (fps)[a]	Snyder-Thellen sarcoma Gardner-Arnstein sarcoma	Cat		TryPK(src)
fgr	Gardner-Rasheed sarcoma	Cat		TyrPK(src)
fms	McDonough sarcoma	Cat		TyrPK CSF-1 receptor
fps (fes)[a]	Fujinami sarcoma	Chicken		TyrPK(src)
fos	FBJ osteosarcoma	Mouse		Nuclear, TR
hst	NVT	Human	Stomach tumor	FGF homologue
intl	NVT	Mouse	MMTV-induced carcinoma	Nuclear, TR
int2	NVT	Mouse	MMTV-induced carcinoma	FGF homologue
jun	ASV17 sarcoma	Chicken		Nuclear, TR
hit	Hardy-Zuckerman 4 sarcoma	Cat		TyrPK GFR L
B-lym	NVT	Chicken	Bursal lymphoma	
mas	NVT	Human	Epidermoid carcinoma	Potentiates response to angiotensin II
met	NVT	Mouse	Osteosarcoma	TyrPK GFR L
mil (raf)[b]	Mill Hill 2 acute leukemia	Chicken		Ser/ThrPK
mos	Moloney sarcoma	Mouse		Ser/ThrPK
myb	Myeloblastosis	Chicken	Leukemia	Nuclear, TR
myc	MC29 myelocytomatosis	Chicken	Lymphomas	Nuclear TR
N-myc	NVT	Human	Neuroblastomas	Nuclear
neu (ErbB2)	NVT	Rat	Neuroblastoma	TryPK GFR L
ral (mil)[b]	3611 sarcoma	Mouse		Ser/ThrPK
Ha-ras	Harvey murine sarcoma	Rat	Bladder, mammary and skin carcinomas	GTP-binding
Ki-ras	Kirsten murine sarcoma	Rat	Lung, colon carcinomas	GTP-binding
N-ras	NVT	Human	Neuroblastomas leukaemias	GTP-binding
rel	Reticuloendothe-liosis	Turkey		
ros	UR2	Chicken		TyrPK GFR L
sis	Simian sarcoma	Monkey		One chain of PDGF
src	Rous sarcoma	Chicken		TyrPK
ski	SKV770	Chicken		Nuclear
trk	NVT	Human	Colon carcinoma	TyrPK GFR L
yes	Y73, Esh sarcoma	Chicken		TyrPK(src)

Similarly, the expression of the following tumor suppressor genes can be monitored by the present methods: p16, p21, p27, p53, RB, WT-1, DCC, NF-1 and APC.

Since abnormally high level of oncogene expression and abnormally low expression of tumor suppressor gene are often good indicators of oncogenesis, the present methods can be used in prognosis or diagnosis of cancer, in monitoring the development of oncogenesis and in evaluating the efficacy of the cancer therapy.

C. Methods to Evaluate a Candidate Protocol or Drug for Treating Disease or Disorder

Since the method of the invention evaluates gene expression with regard to particular control sequences, the effect of various compounds, treatments (such as irradiation) or other perturbations of the genetic environment can be evaluated for their effect on expression using the methods of the invention. Thus, gene toxic agents, for example, can be identified.

In a specific embodiment, a method to evaluate a candidate protocol or drug for treating a disease or disorder is provide herein, which method comprises: a) administering said protocol or drug to a non-human mammalian subject which expresses a fluorophore under the direction of a promoter of a gene associated with a disease or disorder, and determining the expression of said promoter via observing the presence, absence or intensity of the fluorescence generated by said fluorophore at various locations in said mammalian subject by whole-body external fluorescent optical imaging; b) determining the expression of said promoter, via observing the presence, absence or intensity of the fluorescence generated by said fluorophore at various locations in said mammalian subject by whole-body external fluorescent optical imaging, in a control non-human mammalian subject which expresses said fluorophore under the direction of said promoter of said gene; and c) comparing the expression of said promoter determined in steps a) and b), wherein the expression determined in step a) is different from that in step b) identifies said protocol or drug as effective in treating the disease or disorder.

In a preferred embodiment, overexpression of the gene is associated with the disease or disorder and the expression determined in step a) is lower than that in step b) identifies said protocol or drug as effective in treating the disease or disorder.

In another preferred embodiment, underexpression of the gene is associated with the disease or disorder and the expression determined in step a) is higher than that in step b) identifies said protocol or drug as effective in treating the infection.

In still another preferred embodiment, the non-human mammalian subject which expresses a fluorophore under the direction of a promoter of a gene associated with a disease or disorder is produced by delivering a nucleic acid encoding the fluorophore operatively linked to the promoter, or a cell containing the nucleic acid, to the non-human mammalian subject (see Section B supra).

Any non-human mammalian subject can be used in the present screening methods. Preferably, the non-human mammalian subject used in the screening is a well established laboratory animal such as a mice, a rabbit or a non-human primate. Also preferably, the non-human mammalian subject used in the screening is an infectious disease animal model. Still preferably, the non-human mammalian subject used in the screen is a transgenic animal.

Any fluorophores known in the art, including the ones described in Section B, can used in the present screening methods. In a preferred embodiment, the fluorophore used in the screening is s a green fluorescent protein (GFP), a blue fluorescent protein (BFP) or a red fluorescent protein (RFP).

The present methods can be used to screen candidate protocols or drugs for treating any known diseases or disorders. In a preferred embodiment, the diseases or disorders to be screened against are cancers, immune system diseases or disorders, metabolism diseases or disorders, muscle and bone diseases or disorders, nervous system diseases or disorders, signal diseases or disorders and transporter diseases or disorders.

In yet another preferred embodiment, the non-human mammalian subject expresses a fluorophore under the direction of a promoter of an infectious organism and the expression determined in step a) is lower than that in step b) identifies said protocol or drug as effective in treating infection caused by the infectious organism.

The non-human mammalian subject used in the screening may be an infectious disease animal model.

The infectious organism screened against may be a fungus such as a yeast, a bacterium such as an eubacteria or an archaebacteria, or a virus such as a Class I virus, a Class II virus, a Class III virus, a Class IV virus, a Class V virus or a Class VI virus.

Any substances can be screened using the present screening methods for finding drug candidates for treating infection. In a preferred embodiment, a combinatorial library is used in the screening assays. Methods for synthesizing combinatorial libraries and characteristics of such combinatorial libraries are known in the art (See generally, *Combinatorial Libraries: Synthesis, Screening and Application Potential* (Cortese Ed.) Walter de Gruyter, Inc., 1995; Tietze and Lieb, *Curr. Opin. Chem. Biol.,* 2(3):363–71 (1998); Lam, *Anticancer Drug Des.,* 12(3 :145–67 (1997); Blaney and Martin, *Curr. Opin. Chem. Biol.,* 1(1):54–9 (1997); and Schultz and Schultz, *Biotechnol. Prog.,* 12(6 :729–43 (1996)).

If the infection is caused by bacteria, known antibiotics can be screened using the present screening methods for finding a suitable drug candidate. Preferably, the antibiotics to be screened are aminoglycosides (e.g., streptomycin, gentamicin, sisomicin, tobramycin, amicacin), ansamycins (e.g., rifamycin), antimycotics polyenes (e.g., nystatin, pimaricin, amphotericin B., pecilocin), benzofuran derivatives (e.g., griseofulvin), β-lactam antibiotics penicillins (e.g., penicillin G and its derivatives, oral penicillins, penicillinase-fixed penicillin broad-spectrum penicillins, penicillins active against Proteus and Pseudomonas), cephalosporins (e.g., cephalothin, cephaloridine, cephalexin, cefazolin, cefotaxime), chloramphenicol group (e.g., chloramphenicol, thiamphenicol, azidamphenicol), lmidazole fluconazole, itraconazole, linosamides (e.g., lincomycin, clindamycin), macrolides (e.g., azithromycin, erythromycin, oleandomycin, spiramycin, clarithromycin), peptides, peptolides, polypeptides (e.g., polymyxin B and E, bacitracin, tyrothricin, capreomycin, vancomycin), quinolones (e.g., nalidixic acid, ofloxacin, ciprofloxacin, norfioxin), tetracyclines (e.g., tetracycline, oxytetracycline, minocycline, doxycycline) and other antibiotics (e.g., phosphomycin, fusidic acid).

D. Methods to Screen for Gene Expression Modulators and Regulators

The above-described screening methods can also be used to identify gene expression modulators, i.e., trans-acting substances that modulate the expression of a target gene in a multi-cellular organism, or regulators, i.e., cis-acting genes of a multi-cellular organism that regulate the expression of the target gene. Besides for identifying disease or disorder treatment protocols or drugs, the screening methods described herein have wide applications in industrial, agricultural, environmental protection and many other fields. For example, transgenic animals such as transgenic cows are commercially used. It is desirable to find a suitable substance that increases the expression of the transgene and such substance can be added to the animal feed. Similarly, it is desirable to find and modify gene(s) within the transgenic cow that enhances the expression of the target transgene.

Once it is decided that alteration of the expression level of a target gene is desirable, a fluorophore can be operatively linked to the promoter, or other transcriptional control region, of the target gene and be expressed in a multi-cellular organism. Then, the multi-cellular organism expressing the fluorophore can be treated with a test substance to identify which substance modulates the fluorophore expression. Alternatively, the multi-cellular organism expressing the fluorophore itself can be mutagenized to identify genes within itself that alter the fluorophore expression. These screening principles have long been used to identify cis- or trans-acting regulators of gene expression in unicellular organisms such as bacteria or yeast. However, due to the lack of quick and simple screening methods, such screening are impractical for multi-cellular organisms such as plants and animals. The whole-body external optical imaging of gene expression disclosed herein makes such screening or mutant-haunt practical for multi-cellular organisms.

In a specific embodiment, a method to screen for a modulator of the expression of a gene in a multi-cellular organism is provided herein, which method comprises: a) administering a test substance to a non-human multi-cellular organism which expresses a fluorophore under the direction of a promoter of a gene, and determining the expression of said promoter via observing the presence, absence or intensity of the fluorescence generated by said fluorophore at various locations in said multi-cellular organism by whole-body external fluorescent optical imaging; b) determining the expression of said promoter, via observing the presence, absence or intensity of the fluorescence generated by said fluorophore at various locations by whole-body external fluorescent optical imaging, in a control multi-cellular organism which expresses said fluorophore under the direc-

tion of said promoter of said gene; and c) comparing the expression of said promoter determined in steps a) and b), wherein the expression determined in step a) is different from that in step b) identifies said test substance as a modulator of said gene expression. Preferably, the promoter is an endogenous promoter of the multi-cellular organism.

In another specific embodiment, a method to screen for a multi-cellular organism that expresses a gene at an altered level is provided herein, which method comprises: a) administering a mutation-inducing agent or treatment to a non-human multi-cellular organism which expresses a fluorophore under the direction of a promoter of a gene, and determining the expression of said promoter via observing the presence, absence or intensity of the fluorescence generated by said fluorophore at various locations in said multi-cellular organism by whole-body external fluorescent optical imaging; b) determining the expression of said promoter, via observing the presence, absence or intensity of the fluorescence generated by said fluorophore at various locations by whole-body external fluorescent optical imaging, in an untreated control multi-cellular organism which expresses said fluorophore under the direction of said promoter of said gene; and c) comparing the expression of said promoter determined in steps a) and b), wherein the expression determined in step a) is different from that in step b) identifies a multi-cellular organism that expresses said gene at said altered level. Preferably, the mutation-inducing agent or treatment causes a mutation in germ-line cells of the multi-cellular organism so that the desired mutation is stably-transferable to offspring of the multi-cellular organism.

In addition, the various protocols described in the art for "Big Blue" transgenic mice can be utilized in the system of the invention.

The following examples are included for illustrative purposes only and are not intended to limit the scope of the invention.

EXAMPLE 1

Visualization of Gene Expression in Various Tissues using Adenovirus

Four six-week-old male of female nude/nude, nude/+, or C57BL/6 mice were used. All animal studies were conducted in accordance with the principles and procedures outlined in the National Institute of Health Guide for the Care and Use of Animals under assurance number A3873-1. Mice were fed with autoclaved laboratory rodent diet (Tecklad LM-485, Western Research Products, Orange, Calif.).

The vector employed was adenorival (vAd) vector AdCMV5GFPAE1/AE3 [vAd-green fluorescent protein (GFP)] (Quantum, Montreal, Canada), which expresses enhanced GFP and the ampicillin resistance gene.

This vector was provided to various tissues to visualize expression of the CMV promoter in these tissues. Expression of reporter under control of any desired promoter can be visualized by suitable modification of this vector, as described above.

Liver: After exposure of the portal vein following an upper midline abdominal incision, total volume of 100 μl (8×10^{10} pfu/ml) vAd-GFP per mouse were injected in the portal vein using a 1 ml 39G1 latex-free syringe (Becton Dickinson, Franklin Lakes, N.J.). The puncture hole of portal vein was pressed for about 10 seconds with sterile cotton to stop any bleeding. The incision in the abdominal wall was closed with a 7-0 surgical suture in one layer. The animals were kept under Ketamine anesthesia during surgery. All procedures of the operation described above were performed with a 7× magnification microscope (Leica MZ6, Nussloch, Germany). Animals were kept in a barrier facility under HEPA filtration.

Brain: The parietal bone of the skull was exposed after an upper midline scalp incision. Twenty microliters containing 8×10^{10} plaque-forming units (pfu)/ml vAd-GFP per mouse was injected in the brain by using a 1-ml 27G1/2 latex-free syringe (Becton Dickinson). The puncture hole in the skull was plugged with bone wax. The incision in the scalp was closed with a 7-0 surgical suture in one layer. The animals were kept under isofluorane anesthesia during surgery.

Pancreas: The pancreas was exposed after an upper midline abdominal incision. One-hundred microliters containing 8×10^{10} pfu/ml vAd-GFP per mouse was injected in the pancreas by using a 1-ml $30G_{1/2}$ latex-free syringe (Becton Dickinson). The puncture hole was pressed for about 10 sec with sterile cotton for hemostasis. The incision was closed with a 7-0 surgical suture in one layer. The animals were kept under Kersel anesthesia during surgery. All procedures of the operation described above were performed with a ×7 magnification stereo microscope.

Prostate: The bladder and prostate were exposed after a lower midline abdominal incision. Thirty microliters containing 8×10^{10} pfu/ml vAd-GFP per mouse was injected in the prostate by using a 1-ml $30G_{1/2}$ latex-free syringe (Becton Dickinson). The puncture hole in the prostate was pressed for about 10 sec with sterile cotton for hemostasis. The incision in the abdominal wall was closed with a 6-0 surgical suture in one layer. The animals were kept under isofluorane anesthesia during surgery. All procedures of the operation described above were performed with a ×7 magnification stereo miscroscope.

Bone Marrow: For bone marrow injection, animals were anesthetized by inhalation of isofluorane. The skin on the hind leg was opened with a 1-cm incision to expose the tibia. A 27-gauge needle with latex-free syringe (Becton Dickinson) then was inserted in the bone marrow cavity. A total volume of 20 μl (8×10^{10} pfu/ml) vAd-GFP per mouse was injected into the bone marrow cavity. The puncture hole in the bone was plugged with bone wax, and the incision was closed with a 6-0 surgical suture.

Visualization: For visualization at high magnification, Leica fluorescence stereo microscope, model LZ12, equipped with a 50-W mercury lamp, was used. Selective excitation of GFP was produced through a D425/60 band-pass filter and 470 DCXR dichroic mirror. Emitted fluorescence was collected through a long-pass filter GG475 (Chroma Technology, Brattleboro, Vt.) on a Hamamatsu C5810—3-chip cooled color charge-coupled device camera (Hamamatsu Photonics Systems, Bridgewater, N.J.). Images were processed for contrast and brightness and analyzed with the use of IMAGE PRO PLUS 3.1 software (Media Cybernetics, Silver Springs, Md.). Images of 1,024×724 pixels were captured directly on an IBM PC or continuously through video output on a high-resolution Sony VCR model SLV-R-1000 (Sony, Tokyo).

Imaging at lower magnification that visualized the entire animal was carried out in a light box illuminated by blue light fiber optics (Lightools Research, Encinitas, Calif.) and imaged by using the thermoelectrically cooled color charge-coupled device camera, as described above.

Quantitation: The intensity of GFP fluorescence is measured to account for variations in the exciting illumination

with time and across the imaging area. These factors are corrected for by using the intrinsic red fluorescence of mouse skin as a base line to correct the increase over intrinsic green fluorescence caused by GFP. This can be done because there is relatively little red luminance in the GFP radiance. Consequently, the green fluorescence was calculated relative to red based on red and green channel composition in the skin image. A ratio (γ) of green to red channels was determined for each pixel in the image of skin without and with GFP. Values of γ for mouse skin throughout the image in the absence of GFP were fairly constant, varying between 0.7 and 1.0. The contribution of GFP fluorescence from within the animal increased the green component relative to red, which was reflected in higher γ values. The total amount of GFP fluorescence was approximated by multiplying the number of pixels in which value γ was higher than 1 times the γ value of each pixel. Such a product roughly corresponds to the integral GFP fluorescence [Γ_{GFP}] above the maximum value of γ for skin without GFP. The number of pixels in mouse skin images with γ value >1.0 without GFP was less than 0.02% and increased with GFP expression. The value of [Γ_{GFP}] is shown as a function of time after virus injection in FIGS. **1**A and **1**B for brain and liver respectively.

Images of the various organs were compared when taken at high magnification on live intact animals or similar organs viewed directly after death and dissection. The images show the distribution of gene expression in the various organs. In all cases, the images made externally are similar to those of the exposed organs.

When the live animal was viewed in a light box, it was also possible to monitor the expression of the gene, thus permitting a real time observation of the living animal and expression as it occurs in this animal. For example, a light box determination of expression of the GFP in nude mouse liver taken at 72 hours clearly shows this result. Similar results are observed in the nude mouse brain 24 hours after gene delivery. The method is quite sensitive in that the intensity of GFP fluorescence in the mouse liver at a depth of 0.8 mm under the skin was about 25% of that of the exposed organ. Gene expression is externally measurable if the average fluorescence of the GFP expressing organs is at least 20% above the average fluorescence of the surrounding skin, and at maximal level of GFP expression, the intensity in the liver exceeded more than 100 times the back dorsal and abdominal skin fluorescence.

EXAMPLE 2

Visualization of Genes Using Lentiviral Vectors

Lentiviral vectors have been shown to transduce a broad spectrum of non-dividing cells in vitro, such as neurons, retina, liver, muscle and hematopoietic stem cells (see, for example, Naldini, L. et al., *Science* (1996) 272:263–267; Kafri T. et al.; *Nat. Genet* (1997) 17:314–317; Takahashi, M. et al., *J. Virol* (1999) 73:7812–7816; Miyoshi, H. et al. *Science* (1999) 283:682–686). Although it has been reported that hepatocytes are refractory to lentiviral transductioni unless they progress into the cell cycle (Park, F. et al. *Nat. Genet* (2000) 24:49–52), it is shown below that lentiviral gene delivery to the liver for expression visualization is practical.

A lentiviral vector based on HIV1 designated GFP-LV was used. This vector contains a self-inactivating mutation in the U-3 region, a post-transcriptional element, and an internal CMV promoter. It also contains cppt, the central polypurine tract derived from HIV-pol and a woodchuck hepatitis virus post-transcriptional element (WPRE). A diagram of this vector is shown in FIG. **2**A.

The vector GHP-LV at 1×10^9 IU was injected into the portal vein of nude mice; (Hsd:asymic nude-nu). Six (6) days after injection green fluorescence was testable in the liver using in-vivo fluorescence optical imaging, as shown in FIG. **2**B. At day 21, all lobes of the liver of the mice injected with this vector exhibited a homogeneous green fluorescence.

GHP-LV at 1×10^9 IU was also injected intraperitoneally and this method also resulted in a high level of transduction of liver and spleen.

Western Blot demonstrated dose dependence of GFP expression in the range of 0.5–2.5×10^9 IU. Vector integration in the liver 3 weeks after injection was demonstrated by PCR.

Confirmation that the transduced cells were not rapidly dividing was achieved by administering 5' bromo-2' deoxyuridine (BrdU) 15 mgs/kg by daily IP injections in order to label dividing cells. While the cells in the duodenum showed high labelling, only about 3% of liver cells were BrdU positive in either control or lentiviral-treated livers.

EXAMPLE 3

Additional Applications

In addition to the procedures exemplified in Examples 1 and 2, the methods of the invention may be used to monitor expression of control sequences that are regulated by the unfolded protein response (UPR) as described, for example, by Niwa, M., et al., *Cell* (1999) 99:691–702, the contents of which are incorporated herein by reference. Another suitable target for study is the circadian rhythm controlling genes which were studied using less convenient techniques by Yamaguchi, S., et al, *Nature* (2001) 409:684, incorporated herein by reference.

Since modifications will be apparent to those of skill in this art, it is intended that this invention be limited only by the scope of the appended claims.

What is claimed is:

1. A method to monitor the ability of a promoter to promote expression in an animal of an endogenous gene that is controlled by said promoter, which method comprises:

a) delivering, to an animal, cells containing a nucleic acid encoding a fluorophore operatively linked to the promoter of said endogenous gene whose ability to promote expression is to be analyzed; and

b) observing the presence, absence or intensity of the fluorescence generated by said fluorophore at various locations in said animal by whole-body external fluorescent optical imaging,

whereby the ability of said promoter to promote expression is monitored, and

wherein said fluorophore is a protein that is autofluorescent such that no substrates or cofactors are needed for it to fluoresce.

2. The method of claim **1**, wherein the cells are delivered to the animal via a surgical procedure.

3. The method of claim **2**, wherein the cells are delivered to the animal via direct implantation by surgical orthotopic implantation (SOI) at a desired site.

4. The method of claim **1**, wherein the animal is a human and the fluorophore is a humanized fluorophore.

5. The method of claim **1**, wherein the fluorophore is selected from the group consisting of a green fluorescent protein (GFP), a blue fluorescent protein (BFP) and a red fluorescent protein (RFP).

6. The method of claim **5**, wherein the animal is a human and the GFP is the humanized hGFP-S65T.

7. The method of claim **1**, wherein the animal is a mammal.

8. The method of claim **7**, wherein the mammal is selected from the group consisting of a mouse, a rat, a rabbit, a cat, a dog, a pig, a cow, an ox, a sheep, a goat, a horse, a monkey and a non-human primate.

9. The method of claim **1**, wherein the endogenous gene is normally expressed in a tissue or organ specific manner.

10. The method of claim **9**, wherein the tissue is selected from the group consisting of connective, epithelium, muscle and nerve tissues.

11. The method of claim **9**, wherein the organ is selected from the group consisting of brain, lung, liver, spleen, bone marrow, thymus, heart, lymph, blood, bone, cartilage, pancreas, kidney, gall bladder, stomach, intestine, testis, ovary, uterus, rectum, nervous system, gland, and internal blood vessels.

12. The method of claim **1**, wherein the endogenous gene is an endogenous tumor or cancer associated gene.

13. The method of claim **12**, wherein the tumor or cancer associated gene is an oncogene or a tumor suppressor gene.

* * * * *

EXHIBIT 7

EXHIBIT 7

US00RE39337E

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Monosov et al.

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(54) **NUDE MOUSE MODEL FOR HUMAN NEOPLASTIC DISEASE**

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(58) **Field of Classification Search** 800/9, 800/10; 424/93.21
See application file for complete search history.

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(57) **ABSTRACT**

A nude mouse model for human neoplastic diseases having histologically intact human neoplastic tissue transplanted onto an organ of the mouse which corresponds to the human organ from which the tissue is obtained.

50 Claims, No Drawings

NUDE MOUSE MODEL FOR HUMAN NEOPLASTIC DISEASE

Matter enclosed in heavy brackets [ ] appears in the original patent but forms no part of this reissue specification; matter printed in italics indicates the additions made by reissue.

RELATED APPLICATIONS

This application is a continuation of U.S. Ser. No. 08/169,735, filed Dec. 17, 1993, now U.S. Pat. No. 5,491,284, which is a continuation of U.S. Ser. No. 07/719,814 filed Jun. 24, 1991, now abandoned, which is a continuation-in-part of U.S. Ser. No. 253,990 filed Oct. 5, 1988, now abandoned, the contents of which are hereby incorporated by reference in their entirety.

TECHNICAL FIELD

The present invention relates generally to a non-human mammalian model for human neoplastic diseases. More particularly, the invention relates to a non-human mammalian model having neoplastic tissue, obtained from a human organ, transplanted to the corresponding organ of the model.

BACKGROUND

There has long been a need for a representative animal model for human neoplastic disease. Such a model could serve many purposes. For example, it would be used to study the progression of neoplastic disease in human subjects and assist in finding appropriate treatment. Such a model could also be used to test the efficacy of proposed anti-neoplastic agents. Additionally, an animal model could be employed in individualized chemosensitivity testing of a cancer patient's tumors. The existence of such a model would make drug screening, testing and evaluation much more efficient and much less costly.

Some previous attempts at generating animal models for human neoplastic disease employed transplantable animal tumors. There were tumors that had developed in rodents and had been transplanted from animal to animal, usually in inbred populations. Other animal tumor models were generated by inducing tumors in the animals by means of various agents that were carcinogenic, at least in the animal system. Still other animal tumor models were rodents containing spontaneously-occurring tumors. These rodent models, however, frequently responded to chemotherapeutic agents very differently than human subjects receiving the same agent.

Another animal tumor model that developed starting some twenty years ago utilized mice without a thymus gland. These animals were deficient in cellular immunity and had therefore lost their ability to reject foreign transplant tissue. The mice, for reasons not clearly understood, were essentially lacking in hair and came to be called "nude mice" or "athymic T-cell deficient nude mice."

It was found that human tumors often grew when implanted subcutaneously under the skin of nude mice, however, the take rate or frequency with which human tumor tissue actually formed a tumor in the mouse varied depending on the individual donor and the tumor type. In these models, tumors that took exhibited histologically limited invasiveness and rarely metastasized, even if the original human tumor had been highly metastatic. Accordingly, the subcutaneous nude mouse human tumor model, although better than the previously described rodent model, still had substantial drawbacks, i.e. the subcutaneous transplants lacked the ability to metastasize, and also were often more sensitive than the tumor in the patient in the original organ. The differences may be due to the subcutaneous environment regarding pH, vascularity, accessibility to drugs, etc.

Subsequent investigators found that invasion and metastases by human tumor cells in nude mice appeared to require that the cells be implanted orthotopically, i.e. injected into organs involved in the original anatomical environment of the tumor. For example, Wang et al. (Exp. Cell Biology, 50, 330 (1982)) report the expression of malignant phenotype when human colonic tumor cells were implanted by injection within the colonic wall of nude mice. Moreover, Natio et al. (Cancer Research, 46, 4109 (1986)) and Naito et al. (JNCI, 78,377 (1987)) report growth and metastasis of tumor cells isolated from a human renal cell carcinoma and implanted by injection into the kidneys of nude mice. More recently, Morikawa et al. (Cancer Research, 46, 6863 (1988) report the growth of human colon carcinoma cells implanted by injection within the spleens of nude mice.

While the human tumor model created by orthotopic implantation of human tumor cells in the nude mouse represents a significant advance over earlier models, the value of this model is clearly dependent on the extent to which the character of the original human tumor is maintained in the immunodeficient host. Human tumor cells utilized in orthotopic implantation are derived from tumor tissue that is disassociated enzymatically. Enzymatic disassociation disrupts the architecture of the tumor tissue and thus the unique cellular organization. Cells behave very differently when they are organized in a tissue structure as opposed to being disassociated.

Neoplasms are biologically heterogeneous, consisting of different subpopulations of cells having different biological behavior and different metastatic potential (see Naito et al., Cancer Research, 46, 4109–4115 (1986); Naito et al., JNCI, 78,377 (1987); and Morikawa et al., Cancer Research, 48, 6863 {1988}). Enzymatic disassociation of tumor tissue, the conventional method used to isolate tumor cells from fresh surgical specimen, disrupts the original tumor architecture and precludes obtaining a truly representative tumor cell population for implantation. Enzymatic disassociation also alters cellular behavior and drug response.

For example, in routine location of tumor cells for implantation or sensitivity testing, tumor tissue from a surgical specimen is disassociated enzymatically to produce cells which are then implanted subcutaneously (s.c.) in nude mice. The purpose of the s.c. implant is to produce a larger amount of tumor tissue for studies of predictive sensitivity for therapeutic agents as well as for implantation. After sufficient s.c. tumor growth occurs, the tumor is excised and disassociated enzymatically. As mentioned previously, enzymatic disassociation of the tumor cells disrupts the tumor architecture and consequently cells that are selected for sensitivity testing or orthopedic implantation by injection may not be representative or characteristic of the original patient tumor.

Thus the art is presently lacking a truly adequate non-human mammalian model for human neoplastic disease. In particular, what is needed in the art is a model which has the ability to accurately mimic the progression of neoplastic disease as it occurs in a human subject. Such models and methods of generating same are disclosed and claimed herein.

SUMMARY AND OBJECTS OF THE INVENTION

The present invention relates to an improved non-human mammalian model for human neoplastic disease.

In a first aspect, the present invention provides a novel non-human mammalian model for human neoplastic disease wherein histologically intact human neoplastic tissue is transplanted onto the corresponding organ of the model, said model being sufficiently immunodeficient to allow the transplanted tissue to grow and mimic the progression of neoplastic disease in the human donor.

In another aspect, the present invention provides a novel non-human mammalian model for human neoplastic disease wherein neoplastic tissue from a human organ is implanted in a vascularized matrix created on the corresponding organ of the immunodeficient model.

In a further aspect, the present invention provides a novel non-human mammalian model for human neoplastic disease wherein human neoplastic tissue is transplanted to the immunodeficient model by sandwiching the neoplastic tissue between an abdominal skin flap of the model and the corresponding organ of the model.

In yet another aspect, the present invention provides a novel non-human mammalian model for human neoplastic disease wherein neoplastic tissue from a human organ is transplanted to the immunodeficient model by securing, to the surface of the corresponding organ of the model, at least two pieces of neoplastic tissue in close proximity to each other.

In still another aspect, the invention provides a method of generating a non-human mammalian model for human neoplastic disease, the method comprising, providing a laboratory animal having sufficient immunodeficiency to allow implanted human neoplastic tissue to grow and mimic the progression of human neoplastic disease in the donor; by transplanting neoplastic tissue from a human organ into the corresponding organ of the immunodeficient animal.

In yet another aspect, the invention provides a method of generating a non-human mammalian model for human neoplastic disease, the method comprising, providing a laboratory animal having sufficient immunodeficiency to allow implanted human neoplastic tissue to grow and mimic the progression of neoplastic disease in the human donor, securing a vascularizing matrix to a selected organ of the animal and allowing the matrix to vascularize; and implanting neoplastic tissue from a human organ in the vascularized matrix wherein the matrix is located in the corresponding organ of the model.

In still another aspect, the invention provides a method of generating a non-human mammalian model for human neoplastic disease, the method comprising, providing a non-human mammalian laboratory animal having sufficient immunodeficiency to allow implanted human neoplastic tissue to grow and mimic the progression of neoplastic disease in the human donor; and sandwiching neoplastic tissue from a human organ between an abdominal skin flap created in the model and the Corresponding organ of the model.

In yet a further aspect, the invention provides a method of generating a non-human mammalian model from human neoplastic disease, the method comprising, providing a non-human mammalian laboratory animal having sufficient immunodeficiency to allow implanted human neoplastic tissue to grow and mimic the progression of neoplastic disease in the human donor; and securing at least two pieces of neoplastic tissue from a human organ to the surface of the corresponding organ of the model.

DETAILED DESCRIPTION OF THE INVENTION

Copending parent application, U.S. Ser. No. 253,990 filed Oct. 5, 1988, discloses animal models for human neoplastic

disease wherein human neoplastic tissue is implanted into the corresponding organ of an immunodeficient animal that has sufficient immunodeficiency to allow the transplanted neoplastic tissue to grow and mimic the progression of neoplastic disease in the human donor. The method used to generate the animal models disclosed in U.S. Ser. No. 253,990 is described in the following paragraphs and in Examples I, II and III.

Animals that are suitable as immunodeficient hosts includes athymic rodents, i.e. rats and mice having no T-cell immunity. Particularly preferred animals are athymic mice which are readily available and may be obtained commercially from Charles River Laboratories, Inc., Wilmington, Mass. (Catalog identification: Crl:nu/nu(CD-1)BR, homozygous 28–42 days old).

The placement of neoplastic tissue in the immunodeficient host animal according to copending parent application, U.S. Ser. No. 253,990, is carried out by means of orthotopic implantation. This refers to an implant or graft transferred to a position formerly occupied by tissue of the same kind. The terminology orthotopic implantation is used to refer to the grafting of histologically intact human neoplastic tumor tissue from a human organ into the corresponding organ of an immunodeficient animal. Human neoplastic tissue that is utilized comprises tissue from fresh surgical specimens which are pathologically diagnosed tumors occurring in, for example, human kidney, liver, stomach, pancreas, colon, breast, prostate, lung, testis and brain. Such tumors include carcinomas as well as sarcomas and implantation thereof encompasses all stages, grades and types of tumors.

Prior to implantation, the human neoplastic tissue is maintained by placing it in a suitable nutrient medium, such as Eagle's Minimum Essential Medium containing ten percent fetal calf serum and a suitable antibiotic, such as gentamycin. The medium containing the tissue is then cooled to approximately 4° C. Tissue can be maintained in this manner for approximately twenty-four to seventy-two hours.

A selected tissue specimen is prepared for implantation by forming into a mass a suitable size for insertion into a suitably prepared cavity in the selected organ. The specimen size may vary from about 0.1×0.1×0.1 cm to about 0.2×0.1×0.1 cm. The technique used to form a specimen of suitable size comprises testing the tissue to size by pulling into pieces of the desired size with forceps or the like.

Microsurgical instruments typically used to carry out tissue implantation include a castrovijeo needle holder, jeweler's forceps (straight and curved), iris forceps, iris scissors and straight and curved tissue forceps, including one each with teeth and one each without teeth.

Prior to implantation of neoplastic tissue, the selected immunodeficient animal is anesthetized with a suitable anesthetic. Implantation of all organ tissue, except lung tissue, is conveniently accomplished by conventional anesthesia using ethyl ether. When lung tissue is implanted, pentabarbitol is used as the anesthetic.

Implantation of tissue from a hepatoma or tumor from a human liver is carried out utilizing the caudal lobe of the recipient animal's liver as the implantation site. Several loose sutures are placed over the lobe and an incision is made longitudinally under the liver serosa to accommodate a tumor mass of approximately 0.1×0.1×0.1 cm in size. After placement of the tumor mass in the incision, the sutures are pulled snugly over the tumor in order to secure it in place.

The process of implantation of tissue from a human pancreatic tumor is carried out by making an incision in the

recipient animal's pancreas at the head of the organ near the duodenum. Care is exercised to select an a vascular area. An incision is made in the selected area and a tumor mass of approximately 0.1×0.1×0.1 cm is implanted in a manner identical to that described in the preceding paragraph. Tissue from all stages and all grades of pancreatic carcinoma may be implanted in this manner.

The implantation of tissue from a human mammary carcinoma is carried out by surgically implanting the tumor in the mammary fat pad of a recipient female animal. The tumor mass is approximately 0.1×0.1×0.1 cm in size. After placement of the tumor in the pocket, the pocket is closed with a suture. All stages and grades of mammary carcinoma may be implanted in this manner.

Implantation of tissue from a human prostatic carcinoma into the prostate of a recipient animal is carried out by surgically forming an opening in the prostate and then placing 5 tissue specimens of approximately 0.1×0.1×0.1 cm in size under the prostate capsule. After placement of the tissue specimen, the opening in the capsule is closed with appropriate sutures.

Implantation of tissue from a human testicular carcinoma into the testis of a recipient animal is carried out by penetrating the testis along the longitudinal axis with a number-18 gauge needle and injecting a tumor mass of approximately 0.1×0.1×0.1 cm in size through the needle. When the end of the tumor specimen is visible at the tip of the needle, the needle is gently withdrawn while visible tumor tissue is held in place with forceps. The holes made by the needle is then closed with a single suture.

In preparation for implantation of neoplastic lung tissue into the lungs of the recipient animal, a tracheotomy is performed and plastic tubing is incubated. Thereafter, implantation may be effected by several procedures. In one implantation procedure, tracheotomy tubing is advanced to reach either lung lobe(s); a small (0.1×0.1×0.1 cm) tumor mass is injected through the tubing; and the tubing is then removed and the tracheal wound is closed with a suture.

In the other implantation procedure, precautionary tubing is inserted into the trachea; a small stab wound is made on the right chest to bring up a lobe of the right lung which plugs the thoracic cavity thereby preventing collapse of the lung; the lung lobe is gently clamped at the base and two ligatures are loosely placed on the lung; an incision is made on the lung, a tumor mass of approximately 0.1×0.1×0.1 cm is imbedded therein; the ligatures are snugly tied; and the lung lobe is placed back into the thoracic cavity and the wound is closed. Tissue-from all stages and grades of small cell and non-small cell lung carcinomas may be implanted by either of the foregoing procedures.

In order to implant neoplastic human brain tissue into the recipient animal's brain, a bur hole is made through the parietal cranial bone of the animal. A tumor mass of approximately 0.1×0.1×0.1 cm is selected and implanted in the brain. The hole in the cranial bone is then sealed by means of bone wax.

The present invention is an extension and improvement of the invention disclosed in copending parent application U.S. Ser. No. 253,990 filed Oct. 5, 1988. In the present invention, a non-human mammalian model for human neoplastic disease is generated by improved methods of transplanting histologically intact neoplastic tissue from a human organ to the corresponding organ of an immunodeficient model that has sufficient immunodeficiency to allow the transplanted tissue to grow and mimic the progression of neoplastic disease in the human donor. The methods used to generate the animal models of the present invention are described in the following paragraphs and in Examples IV–VII.

Transplantation of neoplastic tissue from a human organ to the corresponding organ of an immunodeficient animal as taught in the present invention is referred to as orthotopic transplantation. In the present invention, the terminology orthotopic transplantation is used to refer to the grafting of histologically intact human neoplastic tumor tissue from a human organ onto the corresponding organ of an immunodeficient animal.

Human neoplastic tissue can be utilized in the present invention as well as preparation of such tissue has been described earlier in accordance with copending parent application U.S. Ser. No. 253,990.

One preferred method for transplanting human neoplastic tissue to an immunodeficient animal model according to the present invention utilizes a vascularizing matrix. The purpose of the matrix is to induce the development of blood vessels and thereby enhance the survival and growth of the transplanted neoplastic tissue. In this method, the matrix is transplanted on the appropriate organ by means of a surgical suture(s). When the matrix becomes well vascularized, which usually occurs in about twenty (20) days, the histologically intact specimen of human neoplastic tissue is implanted directly into the vascularized matrix. The term vascularizing matrix as used herein refers to liquid-permeable, water-insoluble material having the general physical characteristics of a sponge and being substantially absorbable in a living mammalian body. Specific examples of such materials are absorbable gelatin sponge and cellulose sponge. While absorbable gelatin sponge is the preferred vascularizing matrix, those skilled in the art will realize that a number of materials can be utilized as the vascularizing matrix.

Another preferred method of transplanting human neoplastic tissue according to the present invention utilizes an internal skin flap over the transplanted surgical specimen. Use of the skin flap induces vascularization and take of the transplanted tissue. In this method, a U shaped incision is made in the abdomen of the immunodeficient animal model and the resulting skin flap is lifted up and the abdominal wall is opened along the line alba. The cecum (or other organ) is accessed through the abdominal incision, and neoplastic tissue is placed between the cecum serosa (or other organ) and the skin flap. Surgical sutures are applied along the edge of the skin flap to fix the flap to the cecum (or other organ). The cecum (or other organ) together with the skin flap is put back into the abdominal cavity and peritoneum and rectus muscles are closed with sutures. Finally, the skin layer is also closed with sutures and surgical adhesive is applied to ensure a good closure of the abdominal wall.

Still another preferred method of transplanting human neoplastic tissue to an immunodeficient animal model according to the present invention utilizes multiple pieces of tissue arranged in a shish-kabob configuration. In this method, a thread-like material is passed through at least two pieces of human neoplastic tissue and the resulting tissue arrangement is positioned on the surface of the corresponding organ of the immunodeficient mode. The shish-kabob configuration is attached to the animal organ by securing a pair of terminal ends of the thread-like material to the organ. The term thread-like material as used herein refers to absorbable surgical suture such as, for example, Chromic Gut surgical suture and Coated VicrylR surgical suture, both obtainable from Ethicon, Inc. located in Somerville, N.J. A particularly preferred variation of this method of transplan-

tation comprises interspersing pieces of normal tissue between pieces of neoplastic tissue in the shish-kabob configuration.

The animal models of the present invention are particularly useful in studying the progression of human neoplastic disease. These studies, in combination with other clinical testing modalities such as diagnostic imaging, help in the selection of the most appropriate form of treatment.

For example, when an animal model of the present invention is subjected to tumor imaging, the clinician is allowed to identify both primary and secondary sites of tumor growth and to estimate the overall burden of the tumor on the animal. Tumor imaging is conventionally carried out by injecting the animal model with a labeled anti-tumor antibody such as an antibody labeled with a radioactive isotope; allowing the antibody time to localize within the tumor; and then scanning the animal using a radiation detector. When a computer is used to compile an image of the radioactivity detected in the animal's body, the computer can color code the image according to the intensity of the radiation. Zones of high radioactivity in regions of the body not expected to accumulate the antibody or its metabolites indicate the possible present invention.

The animal models of the present invention can also be used to screen new anti-neoplastic agents to determine the ability to such agents to affect tumors at the primary site and also at distant metastatic sites or to prevent distant metastases from occurring. The models will be also useful for individualized chemosensitivity testing of a cancer patient's tumors.

Additionally, the animal models of the present invention are useful in studying the effects of nutrition on the progression of human neoplastic disease. These studies can be particularly significant in view of the demonstrated impact of various deficiencies on healthy subjects.

Examples I–III illustrate the invention which is set forth in copending application U.S. Ser. No. 253,990, filed Oct. 5, 1988. Examples IV–X are provided in order to illustrate the present invention and are not be construed as limiting the scope of the invention or as being inclusive of all embodiments of the invention.

EXAMPLE I

In this example, fresh surgical specimens of tissue from a tumor excised from a human kidney were transplanted into the kidneys of nude mice. The tissue specimens, which were pathologically diagnosed as renal cell carcinoma, were prepared to size by the teasing procedure described earlier.

Five athymic nude mice age four (4) to six (6) weeks were selected as the animal recipients for the implants. In preparation for surgery, the mice were anesthetized with ether. An incision was made in each animal to access the kidney under the capsule. A wedge shaped cavity was formed by excision of the renal cortex of each recipient kidney and a mass of tumor tissue of approximately 0.1×0.1×0.1 cm was placed under the renal capsule. A suture was then employed to secure the implant in place.

The five mice of this example were still alive six months later. Approximately one month following implantation of the tissue, the mice were surgically opened and the implanted tumors were observed. In each case, the tumor was found to have taken, i.e. the implanted neoplastic tissue had invaded adjacent tissue. Histological analysis was performed on the tissue implants at this time. Such analysis comprised removing tissue samples from each animal and comparing the samples with a tissue sample from the tissue donor.

Preparation of the tissue samples for histological analysis was carried out by (1) fixing the sample in formalin; (2) embedding the fixed sample in paraffin; (3) preparing 5-micron sections of the fixed, embedded sample; (4) staining the sections with hematoxylin and cosin; and (5) microscopically observing the tissue structure in each section.

Histological analysis revealed that the tissue in the recipient animals preserved its architecture and tissue type and mimicked progression of the disease in the human donor.

EXAMPLE II

In this example, specimens of human tissue excised from the stomach and pathologically diagnosed as gastric carcinoma were prepared to size by the teasing procedure described earlier.

Five athymic nude mice age four (4) to six (6) weeks were selected as the animal recipients for the implants. In preparation for surgery, the mice were anesthetized with ether.

Each anesthetized mouse was opened to provide access to the stomach. An incision was made in the stomach wall using a number 11 scalpel taking care to penetrate the mucosal layer. A pocket was formed large enough to receive five tumor masses of about 0.1×0.1×0.1 cm each. A tumor piece of approximately this size was selected and inserted into the pocket and the incision was cloned using a 7-0 suture.

The five mice of this example have survived for about three (3) to four (4) months and otherwise appear healthy. Subsequent surgical opening of the stomach of these mice has verified that the tumors have taken.

EXAMPLE III

In this example, specimens of human tissue removed from a human colon and pathologically diagnosed as colon carcinoma were prepared to size by the testing procedure described earlier.

Five athymic nude mice, age four (4) to six (6) weeks were selected as the animal recipients for the implants. In preparation for surgery, the mice were anesthetized with ether. Each anesthetized mouse was opened to provide access to the colon. A pocket or cavity was surgically formed in the seromuscular layer with care exercised not to enter the lumen. Five to ten tumor masses of approximately 0.1×0.1×0.1 cm each were inserted into the pocket which was then closed with a suture.

Four of the five mice which underwent this implant surgery have survived for three to four months and appear to be in a good health. Approximately one month following tissue implantation, the mice were surgically opened and the tumors were observed to have taken.

EXAMPLE IV

This example relates to the use of a vascularizing matrix to induce vascularization and take of orthotopically transplanted human tumor tissue.

A surgical tissue specimen, removed from a human colon and pathologically diagnosed as colon carcinoma, was washed with colon-wash medium. Necrotic tissue was removed and the tumor was then cut into small pieces (about 1-mm^3). Colon-wash medium, used to remove infectious intestinal material, was formulated by combining 500 ml of Minimum Essential Medium with Earle's salts (MEM Earle's) with 70 ml fetal bovine serum, 75.2 mg Penicillin G sodium salt, 125 mg Streptomycin, 10 ml Fungizone antibiotic (250 ug amphotericin B and 205 ug sodium

deoxycholate/ml in deionized distilled water), 5 mg Tetracycline, 50 mg Amikacin, 75 mg chloramphenicol and 50 mg Gentamycin.

GelfoamR brand of absorbable, sterile gelatin sponge (obtained from The Upjohn Co., Kalamazoo, Mich.) was hydrated with MEM Earle's. The hydrated sponge was cut into approximately 0.3–0.5×0.3–0.5×0.3–0.5 cm pieces which were transplanted onto the cecum of nude mice by means of a simple surgical suture on top of the cecum serosa. After 20 days, the sponges became well vascularized.

The transplanted vascularized sponges were cut in the center to make a pocket and about 10–15 of the previously prepared 1-mm^3 tumor pieces were implanted into each pocket which was closed by means of a surgical suture. The tumor grew locally and regional as well as liver metastases occurred.

EXAMPLE V

This example relates to the use of an internal skin flap to induce vascularization and take of orthotopically implanted human tumor tissue.

The tumor tissue used is identical to tissue used in Example IV and was prepared for implantation according to the procedure described in Example IV.

Skin flaps were constructed in the lower abdomen of nude mice by making incisions along three sides of a rectangular area (a U-shaped incision). The flap was lifted up and the abdominal wall was opened along the line alba. The cecum was exteriorized from the abdominal cavity and tumor pieces (about 1-mm^3) were placed between the cecum serosa and the skin flap. Surgical sutures were applied along the two opposing edges of the flap to fix the flap on the cecum. The cecum, together with the skin flap was put back into the abdominal cavity. Peritonerum and rectus muscles were closed with sutures followed by reattachment of the skin flap with sutures. As a last step, surgical adhesive was applied to ensure good closure of the abdominal wall. The tumor grew at the implanted site and formed abdominal metastases.

EXAMPLE VI

This example relates to the use of a shish-kabob tissue configuration to effect orthotopic transplantation of human tumor tissue.

A surgical tissue specimen, removed from a human colon and pathologically diagnosed as colon carcinoma, was well washed with colon-wash medium. Necrotic tissue was removed and the tumor was then cut into small 1-mm^3 pieces. Eight of the 1-mm^3 pieces were assembled in a shish-kabob configuration by stringing the pieces together on a piece of surgical suture.

The shish-kabob tissue configuration was transplanted in nude mice by placement of the configuration on the mouse colon approximately 0.5 to 1 cm away from (i.e., up-stream of) the animals cecum. The configuration is held in place by securing the terminal ends of the suture material to the organ of the animal.

EXAMPLE VII

As shown below, local growth, regional metastasis, and in some instances, distant organ metastasis have been achieved using the three novel methods of orthotopic transplantation described in the present invention.

TABLE 1

Orthotopic Transplantation of Human Colon Carcinoma to the Colon of Nude Mice

Method	Number of Animals	Local Growth	Regional Metastasis	Distant Metastasis
Matrix	3	56–62 days	56–62 days	160 days
Skin Flap	3	48–62 days	48–62 days	
Shish-kabob	4	62–138 days	62–138 days	

EXAMPLE VIII

This example relates to the use of surgical adhesive to glue a piece of tumor tissue onto the top of the urinary bladder of the nude mouse.

A piece of tumor tissue specimen about 2-mm^3 size was prepared for tumor transplantation. The nude mouse was operated on under surgical anesthesia with the full exposure of the urinary bladder. A small amount of surgical adhesive (2-cyanoacrylic acid ester) was applied on the top of the urinary bladder and the previously prepared tumor piece was then glued onto the top of the urinary bladder. The abdomen was closed with surgical sutures.

Using the transplantation method described above, we transplanted the ras-transfected human bladder RT-10 carcinoma cell line xenograft. As a result, we achieved unexpected extensive growth and metastases, including invasion of the whole thickness of the urinary bladder wall, lymph node metastases, and multi-organ metastases in the liver, pancreas, spleen, ovary, kidney, ureter and lung. This transplantation result of RT-10 is in striking contrast to the result obtained when RT-10 was injected transurethrally as disaggregated cells where only local invasion and no distant metastasis were observed. (Theodorescu et al., Proc. Natl. Acad. Sci(1990), Vol 87, 9047–9051.)

EXAMPLE IX

In this example, a human tongue cancer specimen, which was prepared by being cut into 1-mm^3 pieces, was transplanted orthotopically to the floor of the mouth of a nude mouse.

An incision was made along the midline on the upper neck of the mouse. After blunt dissection of the muscles of the floor of the mouth, five pieces of prepared tumor tissue were implanted in between the muscles deep in the floor of the mouth. Surgical sutures were applied to close the dissected muscles and skin layer.

Extremely invasive growth was observed which involved the whole jaw as well as deep in the nasopharynx. This is distinctly different form growth observed when tumor pieces were implanted subcutaneously in the neck area. The subcutaneously grown tumor was completely encapsulated and exhibited no invasion of the adjacent tissue.

EXAMPLE X

In this example, a human pancreatic tumor specimen was prepared by being cut into 1-mm^3 pieces, and tumor pieces were transplanted onto the nude mouse pancreas.

A midline incision was made on the upper abdomen of the nude mouse muscle layers and the peritoneum were opened along the line alba. Ten (10) pieces of previously prepared tumor pieces were assembled in a shish-kebab configuration

by striking the pieces together on a piece of surgical suture. The configuration was secured on the pancreas. The abdomen was then closed with one layer surgical suture.

Two different kinds of human pancreatic cell line xenografts were transplanted as described above. Invasive growth was observed into both cases, including the invasion of the duodenum and spleen.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

What is claimed is:

1. A nude mouse model for *progression of* human neoplastic disease*, the progression of said disease being characterized by growth of a primary tumor site and metastasis to secondary tumor sites,* wherein said mouse [is characterized by

having] *has* histologically intact human neoplastic tissue of at least 1 mm^3 in size transplanted onto an organ of said mouse which corresponds to the human organ from which said tissue is originally obtained; and

[having] *has* sufficient immuno-deficiency to allow said transplanted neoplastic tissue to grow [and] *at said primary site and metastasize to said secondary tumor sites, so as to* mimic the progression of the neoplastic disease *including the metastatic behavior of said neoplastic disease* in [the human donor] *humans*.

2. A nude mouse model according to claim **1** wherein said neoplastic tissue is selected from breast tissue, ovarian tissue [or] *and* pleural tissue.

3. A nude mouse model according to claim **2** wherein said neoplastic tissue is obtained from human breast tissue.

4. A nude mouse model according to claim **3** wherein said human neoplastic breast tissue is implanted in the mammary fat pad of the mouse.

5. A nude mouse model according to claim **2** wherein said neoplastic tissue is obtained from human ovarian tissue.

6. A nude mouse model according to claim **5** wherein said human neoplastic ovarian tissue is implanted in the ovarian capsule of the mouse.

7. A nude mouse model according to claim **5** wherein said human neoplastic ovarian tissue is transplanted by securing to the surface of the mouse ovary at least two pieces of neoplastic tissue in close proximity to each other.

8. A nude mouse model according to claim **2** wherein said neoplastic tissue is obtained from human pleural tissue.

9. A nude mouse model according to claim **8** wherein said neoplastic tissue is implanted in the parietal pleura of the mouse.

10. A nude mouse model according to claim **9** wherein said neoplastic tissue is implanted in the visceral pleura of the mouse.

11. A method of generating a nude mouse model for *progression of* human neoplastic disease, *the progression of said disease being characterized by growth of a primary tumor site and metastasis to secondary tumor sites,* said method comprising:

transplanting histologically intact human neoplastic tissue of at least 1 mm^3 in size [transplanted] onto an organ of a nude mouse which corresponds to the human organ from which said tissue is originally obtained; and

allowing said transplanted tissue to grow [and] *at said primary site and metastasize to said secondary tumor sites, so as to* mimic progression of the neoplastic disease *including the metastatic behavior of said neoplastic disease* in [the human donor] *humans*.

12. A method of generating a nude mouse according to claim **11** wherein said human neoplastic tissue is selected from breast tissue, ovarian tissue [or] *and* pleural tissue.

13. A nude rodent model for progression of human neoplastic disease, the progression of said disease being characterized by growth of a primary tumor site and metastasis to secondary tumor sites, wherein said rodent has histologically intact human neoplastic tissue of at least 1 mm^3 in size transplanted onto an organ of said rodent which corresponds to the human organ from which said tissue is originally obtained; and

has sufficient immuno-deficiency to allow said transplanted neoplastic tissue to grow at said primary site and metastasize to said secondary tumor sites, so as to mimic the progression of the neoplastic disease including the metastatic behavior of said neoplastic disease in humans.

14. The nude rodent model for human neoplastic disease of claim 13, wherein said rodent is a rat.

15. An immunodeficient rodent model for progression of human neoplastic disease, the progression of said disease being characterized by growth of a primary tumor site and metastasis to secondary tumor sites, wherein said rodent has histologically intact human neoplastic tissue of at least 1 mm^3 in size transplanted onto an organ of said rodent which corresponds to the human organ from which said tissue is originally obtained; and

has sufficient immuno-deficiency to allow said transplanted neoplastic tissue to grow at said primary site and metastasize to said secondary tumor sites, so as to mimic the progression of the neoplastic disease including the metastatic behavior of said neoplastic disease in humans.

16. The immunodeficient rodent model for human neoplastic disease of claim 15, wherein said rodent is a rat.

17. The immunodeficient rodent model for human neoplastic disease of claim 15, wherein said rodent is a mouse.

18. The immunodeficient rodent model for human neoplastic disease of claim 17, wherein said rodent is a severe combined immunodeficient (SCID) mouse.

19. A method of generating a nude rodent model for progression of human neoplastic disease, the progression of said disease being characterized by growth of a primary tumor site and metastasis to a secondary tumor sites, said method comprising:

transplanting histologically intact human neoplastic tissue of at least 1 mm^3 in size onto an organ of a nude rodent which corresponds to the human organ from which said tissue is originally obtained; and

allowing said transplanted tissue to grow at said primary site and metastasize to said secondary tumor sites, so as to mimic progression of the neoplastic disease including the metastatic behavior of said neoplastic disease in humans.

20. The method of generating a nude rodent model for human neoplastic disease of claim 19, wherein said rodent is a rat.

21. A method of generating an immunodeficient rodent model for progression of human neoplastic disease, the progression of said disease being characterized by growth of a primary tumor site and metastasis to secondary tumor sites, said method comprising:

transplanting histologically intact human neoplastic tissue of at least 1 mm^3 in size onto an organ of an immunodeficient rodent which corresponds to the human organ from which said tissue is originally obtained; and

allowing said transplanted tissue to grow at said primary site and metastasize to said secondary tumor sites, so as to mimic progression of the neoplastic disease including the metastatic behavior of said neoplastic disease in humans.

22. The method of generating an immunodeficient rodent model for human neoplastic disease of claim 21, wherein said rodent is a rat.

23. The method of generating an immunodeficient rodent model for human neoplastic disease of claim 21, wherein said rodent is a mouse.

24. The method of generating an immunodeficient rodent model for human neoplastic disease of claim 23, wherein said rodent is a severe combined immunodeficient (SCID) mouse.

25. A nude rodent model for progression of human neoplastic disease, the progression of said disease being characterized by growth of a primary tumor site and metastasis to secondary tumor sites, wherein said rodent has histologically intact human neoplastic tissue transplanted onto an organ of said rodent which corresponds to the human organ from which said tissue is originally obtained; and

has sufficient immuno-deficiency to allow said transplanted neoplastic tissue to grow at said primary site and metastasize to said secondary tumor sites, so as to mimic the progression of the neoplastic disease including the metastatic behavior of said neoplastic disease in humans.

26. An immunodeficient rodent model for progression of human neoplastic disease, the progression of said disease being characterized by growth of a primary tumor site and metastasis to secondary tumor sites, wherein said rodent has histologically intact human neoplastic tissue transplanted onto an organ of said rodent which corresponds to the human organ from which said tissue is originally obtained; and

has sufficient immuno-deficiency to allow said transplanted neoplastic tissue to grow at said primary site and metastasize to said secondary tumor sites, so as to mimic the progression of the neoplastic disease including the metastatic behavior of said neoplastic disease in humans.

27. A nude rodent model according to claim 13 wherein said neoplastic tissue is selected from breast tissue, ovarian tissue or pleural tissue.

28. A nude rodent model according to claim 27 wherein said neoplastic tissue is obtained from human breast tissue.

29. A nude rodent model according to claim 27 wherein said neoplastic tissue is obtained from human ovarian tissue.

30. A nude rodent model according to claim 27 wherein said neoplastic tissue is obtained from human pleural tissue.

31. An immunodeficient rodent model according to claim 15 wherein said neoplastic tissue is selected from breast tissue, ovarian tissue or pleural tissue.

32. An immunodeficient rodent model according to claim 31 wherein said neoplastic tissue is obtained from human breast cancer.

33. An immunodeficient rodent model according to claim 31 wherein said neoplastic tissue is obtained from human ovarian tissue.

34. An immunodeficient rodent model according to claim 31 wherein said neoplastic tissue is obtained from human pleural tissue.

35. The method according to claim 19 wherein said neoplastic tissue is selected from breast tissue, ovarian tissue or pleural tissue.

36. The method according to claim 35 wherein said neoplastic tissue is obtained from human breast tissue.

37. The method according to claim 35 wherein said neoplastic tissue is obtained from human ovarian tissue.

38. The method according to claim 35 wherein said neoplastic tissue is obtained from human pleural tissue.

39. The method according to claim 21 wherein said neoplastic tissue is selected from breast tissue, ovarian tissue or pleural tissue.

40. The method according to claim 39 wherein said neoplastic tissue is obtained from human breast tissue.

41. The method according to claim 39 wherein said neoplastic tissue is obtained from human ovarian tissue.

42. The method according to claim 39 wherein said neoplastic tissue is obtained from human pleural tissue.

43. A nude rodent model according to claim 25 wherein said neoplastic tissue is selected from breast tissue, ovarian tissue or pleural tissue.

44. A nude rodent model according to claim 43 wherein said neoplastic tissue is obtained from human breast tissue.

45. A nude rodent model according to claim 43 wherein said neoplastic tissue is obtained from human ovarian tissue.

46. A nude rodent model according to claim 43 wherein said neoplastic tissue is obtained from human pleural tissue.

47. An immunodeficient rodent model according to claim 26 wherein said neoplastic tissue is selected from breast tissue, ovarian tissue or pleural tissue.

48. An immunodeficient rodent model according to claim 47 wherein said neoplastic tissue is obtained from human breast tissue.

49. An immunodeficient rodent model according to claim 47 wherein said neoplastic tissue is obtained from human ovarian tissue.

50. An immunodeficient rodent model according to claim 47 wherein said neoplastic tissue is obtained from human pleural tissue.

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United States Patent [19]

Monosov et al.

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[45] Date of Patent: * Oct. 29, 1996

[54] **NUDE MOUSE MODEL FOR HUMAN NEOPLASTIC DISEASE**

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[*] Notice: The term of this patent shall not extend beyond the expiration date of Pat. No. 5,491,284.

[21] Appl. No.: **459,730**

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Related U.S. Application Data

[63] Continuation of Ser. No. 169,735, Dec. 17, 1993, Pat. No. 5,491,284, which is a continuation of Ser. No. 719,814, Jun. 24, 1991, abandoned, which is a continuation-in-part of Ser. No. 253,990, Oct. 5, 1988, abandoned.

[51] **Int. Cl.**^{6} **A61K 35/00**; A61K 49/00

[52] **U.S. Cl.** **800/2**; 800/DIG. 5; 424/573; 424/559; 424/574; 424/557; 424/9.2

[58] **Field of Search** .... 800/2, DIG. 5; 424/573, 574, 559, 551, 556, 557, 9.2

[56] **References Cited**

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[57] **ABSTRACT**

A nude mouse model for human neoplastic disease having histologically intact human neoplastic tissue transplanted onto an organ of the mouse which corresponds to the human organ from which the tissue is obtained.

12 Claims, No Drawings

NUDE MOUSE MODEL FOR HUMAN NEOPLASTIC DISEASE

RELATED APPLICATIONS

This application is a continuation of U.S. Ser. No. 08/169,735, filed Dec. 17, 1993, now U.S. Pat. No. 5,491,284, which is a continuation of U.S. Ser. No. 07/719,814 filed Jun. 24, 1991, now abandoned, which is a continuation-in-part of U.S. Ser. No. 253,990 filed Oct. 5, 1988, now abandoned, the contents of which are hereby incorporated by reference in their entirety.

TECHNICAL FIELD

The present invention relates generally to a non-human mammalian model for human neoplastic disease. More particularly, the invention relates to a non-human mammalian model having neoplastic tissue, obtained from a human organ, transplanted to the corresponding organ of the model.

BACKGROUND

There has long been a need for a representative animal model for human neoplastic disease. Such a model could serve many purposes. For example, it could be used to study the progression of neoplastic disease in human subjects and assist in finding appropriate treatment. Such a model could also be used to test the efficacy of proposed anti-neoplastic agents. Additionally, an animal model could be employed in individualized chemosensitivity testing of a cancer patient's tumors. The existence of such a model would make drug screening, testing and evaluation much more efficient and much less costly.

Some previous attempts at generating animal models for human neoplastic disease employed transplantable animal tumors. These were tumors that had developed in rodents and had been transplanted from animal to animal, usually in inbred populations. Other animal tumor models were generated by inducing tumors in the animals by means of various agents that were carcinogenic, at least in the animal system. Still other animal tumor models were rodents containing spontaneously-occurring tumors. These rodent models, however, frequently responded to chemotherapeutic agents very differently than human subjects receiving the same agent.

Another animal tumor model that developed starting some twenty years ago utilized mice without a thymus gland. These animals were deficient in cellular immunity and had therefore lost their ability to reject foreign transplant tissue. The mice, for reasons not clearly understood, were essentially lacking in hair and came to be called "nude mice" or "athymic T-cell deficient nude mice."

It was found that human tumors often grew when implanted subcutaneously under the skin of nude mice, however, the take rate or frequency with which human tumor tissue actually formed a tumor in the mouse varied depending on the individual donor and the tumor type. In these models, tumors that took exhibited histologically limited invasiveness and rarely metastasized, even if the original human tumor had been highly metastatic. Accordingly, the subcutaneous nude mouse human tumor model, although better than the previously described rodent model, still had substantial drawbacks, i.e. the subcutaneous transplants lacked the ability to metastasize, and also were often more sensitive than the tumor in the patient in the original organ.

The differences may be due to the subcutaneous environment regarding pH, vascularity, accessibility to drugs, etc.

Subsequent investigators found that invasion and metastases by human tumor cells in nude mice appeared to require that the cells be implanted orthotopically, i.e. injected into organs involved in the original anatomical environment of the tumor. For example, Wang et al. (Exp. Cell Biology, 50, 330 (1982)) report the expression of malignant phenotype when human colonic tumor cells were implanted by injection within the colonic wall of nude mice. Moreover, Naito et al. (Cancer Research, 46, 4109 (1986)) and Naito et al. (JNCI, 78,377 (1987)) report growth and metastasis of tumor cells isolated from a human renal cell carcinoma and implanted by injection into the kidneys of nude mice. More recently, Morikawa et al. (Cancer Research, 46, 6863 (1988) report the growth of human colon carcinoma cells implanted by injection within the spleens of nude mice.

While the human tumor model created by orthotopic implantation of human tumor cells in the nude mouse represents a significant advance over earlier models, the value of this model is clearly dependent on the extent to which the character of the original human tumor is maintained in the immunodeficient host. Human tumor cells utilized in orthotopic implantation are derived from tumor tissue that is disassociated enzymatically. Enzymatic disassociation disrupts the architecture of the tumor tissue and thus the unique cellular organization. Cells behave very differently when they are organized in a tissue structure as opposed to being disassociated.

Neoplasms are biologically heterogeneous, consisting of different subpopulations of cells having different biological behavior and different metastatic potential (see Naito et al., Cancer Research, 46, 4109–4115 (1986); Naito et al., JNCI, 78,377 (1987); and Morikawa et al., Cancer Research, 48, 6863 {1988}). Enzymatic disassociation of tumor tissue, the conventional method used to isolate tumor cells from fresh surgical specimens, disrupts the original tumor architecture and precludes obtaining a truly representative tumor cell population for implantation. Enzymatic disassociation also alters cellular behavior and drug response.

For example, in routine isolation of tumor cells for implantation or sensitivity testing, tumor tissue from a surgical specimen is disassociated enzymatically to produce cells which are then implanted subcutaneously (s.c.) in nude mice. The purpose of the s.c. implant is to produce a larger amount of tumor tissue for studies of predictive sensitivity for therapeutic agents as well as for implantation. After sufficient s.c. tumor growth occurs, the tumor is excised and disassociated enzymatically. As mentioned previously, enzymatic disassociation of the tumor cells disrupts the tumor architecture and consequently cells that are selected for sensitivity testing or orthotopic implantation by injection may not be representative or characteristic of the original patient tumor.

Thus the art is presently lacking a truly adequate non-human mammalian model for human neoplastic disease. In particular, what is needed in the art is a model which has the ability to accurately mimic the progression of neoplastic disease as it occurs in a human subject. Such models and methods of generating same are disclosed and claimed herein.

SUMMARY AND OBJECTS OF THE INVENTION

The present invention relates to an improved non-human mammalian model for human neoplastic disease.

In a first aspect, the present invention provides a novel non-human mammalian model for human neoplastic disease wherein histologically intact human neoplastic tissue is transplanted onto the corresponding organ of the model, said model being sufficiently immunodeficient to allow the transplanted tissue to grow and mimic the progression of neoplastic disease in the human donor.

In another aspect, the present invention provides a novel non-human mammalian model for human neoplastic disease wherein neoplastic tissue from a human organ is implanted in a vascularized matrix created on the corresponding organ of the immunodeficient model.

In a further aspect, the present invention provides a novel non-human mammalian model for human neoplastic disease wherein human neoplastic tissue is transplanted to the immunodeficient model by sandwiching the neoplastic tissue between an abdominal skin flap of the model and the corresponding organ of the model.

In yet another aspect, the present invention provides a novel non-human mammalian model for human neoplastic disease wherein neoplastic tissue from a human organ is transplanted to the immunodeficient model by securing, to the surface of the corresponding organ of the model, at least two pieces of neoplastic tissue in close proximity to each other.

In still another aspect, the invention provides a method of generating a non-human mammalian model for human neoplastic disease, the method comprising, providing a laboratory animal having sufficient immunodeficiency to allow implanted human neoplastic tissue to grow and mimic the progression of human neoplastic disease in the donor; by transplanting neoplastic tissue from a human organ into the corresponding organ of the immunodeficient animal.

In yet another aspect, the invention provides a method of generating a non-human mammalian model for human neoplastic disease, the method comprising, providing a laboratory animal having sufficient immunodeficiency to allow implanted human neoplastic tissue to grow and mimic the progression of neoplastic disease in the human donor; securing a vascularizing matrix to a selected organ of the animal and allowing the matrix to vascularize; and implanting neoplastic tissue from a human organ in the vascularized matrix wherein the matrix is located in the corresponding organ of the model.

In still another aspect, the invention provides a method of generating a non-human mammalian model for human neoplastic disease, the method comprising, providing a non-human mammalian laboratory animal having sufficient immunodeficiency to allow implanted human neoplastic tissue to grow and mimic the progression of neoplastic disease in the human donor; and sandwiching neoplastic tissue from a human organ between an abdominal skin flap created in the model and the Corresponding organ of the model.

In yet a further aspect, the invention provides a method of generating a non-human mammalian model for human neoplastic disease, the method comprising, providing a non-human mammalian laboratory animal having sufficient immunodeficiency to allow implanted human neoplastic tissue to grow and mimic the progression of neoplastic disease in the human donor; and securing at least two pieces of neoplastic tissue from a human organ to the surface of the corresponding organ of the model.

DETAILED DESCRIPTION OF THE INVENTION

Copending parent application, U.S. Ser. No. 253,990 filed Oct. 5, 1988, discloses animal models for human neoplastic disease wherein human neoplastic tissue is implanted into the corresponding organ of an immunodeficient animal that has sufficient immunodeficiency to allow the transplanted neoplastic tissue to grow and mimic the progression of neoplastic disease in the human donor. The method used to generate the animal models disclosed in U.S. Ser. No. 253,990 is described in the following paragraphs and in Examples I, II and III.

Animals that are suitable as immunodeficient hosts include athymic rodents, i.e. rats and mice having no T-cell immunity. Particularly preferred animals are athymic mice which are readily available and may be obtained commercially from Charles River Laboratories, Inc., Wilmington, Mass. (Catalog identification: Crl:nu/nu(CD-1)BR, homozygous 28–42 days old).

The placement of neoplastic tissue in the immunodeficient host animal according to copending parent application, U.S. Ser. No. 253,990, is carried out by means of orthotopic implantation. This refers to an implant or graft transferred to a position formerly occupied by tissue of the same kind. The terminology orthotopic implantation is used to refer to the grafting of histologically intact human neoplastic tumor tissue from a human organ into the corresponding organ of an immunodeficient animal. Human neoplastic tissue that is utilized comprises tissue from fresh surgical specimens which are pathologically diagnosed tumors occurring in, for example, human kidney, liver, stomach, pancreas, colon, breast, prostate, lung, testis and brain. Such tumors include carcinomas as well as sarcomas and implantation thereof encompasses all stages, grades and types of tumors.

Prior to implantation, the human neoplastic tissue is maintained by placing it in a suitable nutrient medium, such as Eagle's Minimum Essential Medium containing ten percent fetal calf serum and a suitable antibiotic, such as gentamycin. The medium containing the tissue is then cooled to approximately 4° C. Tissue can be maintained in this manner for approximately twenty-four to seventy-two hours.

A selected tissue specimen is prepared for implantation by forming into a mass a suitable size for insertion into a suitably prepared cavity in the selected organ. The specimen size may vary from about 0.1×0.1×0.1 cm to about 0.2×0.1×0.1 cm. The technique used to form a specimen of suitable size comprises teasing the tissue to size by pulling into pieces of the desired size with forceps or the like.

Microsurgical instruments typically used to carry out tissue implantation include a castrovijeo needle holder, jeweler's forceps (straight and curved), iris forceps, iris scissors and straight and curved tissue forceps, including one each with teeth and one each without teeth.

Prior to implantation of neoplastic tissue, the selected immunodeficient animal is anesthetized with a suitable anesthetic. Implantation of all organ tissue, except lung tissue, is conveniently accomplished by conventional anesthesia using ethyl ether. When lung tissue is implanted, pentabarbitol is used as the anesthetic.

Implantation of tissue from a hepatoma or tumor from a human liver is carried out utilizing the caudal lobe of the recipient animal's liver as the implantation site. Several loose sutures are placed over the lobe and an incision is made longitudinally under the liver serosa to accommodate a tumor mass of approximately 0.1×0.1 0.1 cm in size. After placement of the tumor mass in the incision, the sutures are pulled snugly over the tumor in order to secure it in place.

The process of implantation of tissue from a human pancreatic tumor is carried out by making an incision in the

recipient animal's pancreas at the head of the organ near the duodenum. Care is exercised to select an avascular area. An incision is made in the selected area and a tumor mass of approximately 0.1×0.1×0.1 cm is implanted in a manner identical to that described in the preceding paragraph. Tissue from all stages and all grades of pancreatic carcinoma may be implanted in this manner.

The implantation of tissue from a human mammary carcinoma is carried out by surgically implanting the tumor in the mammary fat pad of a recipient female animal. The tumor mass is approximately 0.1×0.1×0.1 cm in size. After placement of the tumor in the pocket, the pocket is closed with a suture. All stages and grades of mammary carcinoma may be implanted in this manner.

Implantation of tissue from a human prostatic carcinoma into the prostate of a recipient animal is carried out by surgically forming an opening in the prostate and then placing 5 tissue specimens of approximately 0.1×0.1×0.1 cm in size under the prostate capsule. After placement of the tissue specimen, the opening in the capsule is closed with appropriate sutures.

Implantation of tissue from a human testicular carcinoma into the testis of a recipient animal is carried out by penetrating the testis along the longitudinal axis with a number-18 gauge needle and injecting a tumor mass of approximately 0.1×0.1×0.1 cm in size through the needle. When the end of the tumor specimen is visible at the tip of the needle, the needle is gently withdrawn while visible tumor tissue is held in place with forceps. The hole made by the needle is then closed with a single suture.

In preparation for implantation of neoplastic lung tissue into the lungs of the recipient animal, a tracheotomy is performed and plastic tubing is intubated. Thereafter, implantation may be effected by several procedures. In one implantation procedure, tracheotomy tubing is advanced to reach either lung lobe(s); a small (0.1×0.1×0.1 cm) tumor mass is injected through the tubing; and the tubing is then removed and the tracheal wound is closed with a suture.

In the other implantation procedure, precautionary tubing is inserted into the trachea; a small stab wound is made on the right chest to bring up a lobe of the right lung which plugs the thoracic cavity thereby preventing collapse of the lung; the lung lobe is gently clamped at the base and two ligatures are loosely placed on the lung; an incision is made on the lung, a tumor mass of approximately 0.1×0.1×0.1 cm is imbedded therein; the ligatures are snugly tied; and the lung lobe is placed back into the thoracic cavity and the wound is closed. Tissue-from all stages and grades of small cell and non-small cell lung carcinomas may be implanted by either of the foregoing procedures.

In order to implant neoplastic human brain tissue into the recipient animal's brain, a bur hole is made through the parietal cranial bone of the animal. A tumor mass of approximately 0.1×0.1×0.1 cm is selected and implanted in the brain. The hole in the cranial bone is then sealed by means of bone wax.

The present invention is an extension and improvement of the invention disclosed in copending parent application U.S. Ser. No. 253,990 filed Oct. 5, 1988. In the present invention, a non-human mammalian model for human neoplastic disease is generated by improved methods of transplanting histologically intact neoplastic tissue from a human organ to the corresponding organ of an immunodeficient model that has sufficient immunodeficiency to allow the transplanted tissue to grow and mimic the progression of neoplastic disease in the human donor. The methods used to generate the animal models of the present invention are described in the following paragraphs and in Examples IV–VII.

Transplantation of neoplastic tissue from a human organ to the corresponding organ of an immunodeficient animal as taught in the present invention is referred to as orthotopic transplantation. In the present invention, the terminology orthotopic transplantation is used to refer to the grafting of histologically intact human neoplastic tumor tissue from a human organ onto the corresponding organ of an immunodeficient animal.

Human neoplastic tissue that can be utilized in the present invention as well as preparation of such tissue has been described earlier in connection with copending parent application U.S. Ser. No. 253,990.

One preferred method for transplanting human neoplastic tissue to an immunodeficient animal model according to the present invention utilizes a vascularizing matrix. The purpose of the matrix is to induce the development of blood vessels and thereby enhance the survival and growth of the transplanted neoplastic tissue. In this method, the matrix is transplanted on the appropriate organ by means of a surgical suture(s). When the matrix becomes well vascularized, which usually occurs in about twenty (20) days, the histologically intact specimen of human neoplastic tissue is implanted directly into the vascularized matrix. The term vascularizing matrix as used herein refers to liquid-permeable, water-insoluble material having the general physical characteristics of a sponge and being substantially absorbable in a living mammalian body. Specific examples of such materials are absorbable gelatin sponge and cellulose sponge. While absorbable gelatin sponge is the preferred vascularizing matrix, those skilled in the art will realize that a number of materials can be utilized as the vascularizing matrix.

Another preferred method of transplanting human neoplastic tissue according to the present invention utilizes an internal skin flap over the transplanted surgical specimen. Use of the skin flap induces vascularization and take of the transplanted tissue. In this method, a U shaped incision is made in the abdomen of the immunodeficient animal model and the resulting skin flap is lifted up and the abdominal wall is opened along the linea alba. The cecum (or other organ) is accessed through the abdominal incision, and neoplastic tissue is placed between the cecum serosa (or other organ) and the skin flap. Surgical sutures are applied along the edge of the skin flap to fix the flap to the cecum (or other organ). The cecum (or other organ) together with the skin flap is put back into the abdominal cavity and peritoneum and rectus muscles are closed with sutures. Finally, the skin layer is also closed with sutures and surgical adhesive is applied to ensure a good closure of the abdominal wall.

Still another preferred method of transplanting human neoplastic tissue to an immunodeficient animal model according to the present invention utilizes multiple pieces of tissue arranged in a shish-kabob configuration. In this method, a thread-like material is passed through at least two pieces of human neoplastic tissue and the resulting tissue arrangement is positioned on the surface of the corresponding organ of the immunodeficient model. The shish-kabob configuration is attached to the animal organ by securing a pair of terminal ends of the thread-like material to the organ. The term thread-like material as used herein refers to absorbable surgical suture such as, for example, Chromic Gut surgical suture and Coated VicrylR surgical suture, both obtainable from Ethicon, Inc. located in Somerville, N.J. A particularly preferred variation of this method of transplan-

tation comprises interspersing pieces of normal tissue between pieces of neoplastic tissue in the shish-kabob configuration.

The animal models of the present invention are particularly useful in studying the progression of human neoplastic disease. These studies, in combination with other clinical testing modalities such as diagnostic imaging, help in the selection of the most appropriate form of treatment.

For example, when an animal model of the present invention is subjected to tumor imaging, the clinician is allowed to identify both primary and secondary sites of tumor growth and to estimate the overall burden of the tumor on the animal. Tumor imaging is conventionally carried out by injecting the animal model with a labeled anti-tumor antibody such as an antibody labeled with a radioactive isotope; allowing the antibody time to localize within the tumor; and then scanning the animal using a radiation detector. When a computer is used to compile an image of the radioactivity detected in the animal's body, the computer can color code the image according to the intensity of the radiation. Zones of high radioactivity in regions of the body not expected to accumulate the antibody or its metabolites indicate the possible presence of tumors.

The animal models of the present invention can also be used to screen new anti-neoplastic agents to determine the ability of such agents to affect tumors at the primary site and also at distant metastatic sites or to prevent distant metastases from occurring. The models will be also useful for individualized chemosensitivity testing of a cancer patient's tumors.

Additionally, the animal models of the present invention are useful in studying the effects of nutrition on the progression of human neoplastic disease. These studies can be particularly significant in view of the demonstrated impact of various deficiencies on healthy subjects.

Examples I–III illustrate the invention which is set forth in copending application U.S. Ser. No. 253,990, filed Oct. 5, 1988. Examples IV–X are provided in order to illustrate the present invention and are not to be construed as limiting the scope of the invention or as being inclusive of all embodiments of the invention.

EXAMPLE I

In this example, fresh surgical specimens of tissue from a tumor excised from a human kidney were transplanted into the kidneys of nude mice. The tissue specimens, which were pathologically diagnosed as renal cell carcinoma, were prepared to size by the teasing procedure described earlier.

Five athymic nude mice age four (4) to six (6) weeks were selected as the animal recipients for the implants. In preparation for surgery, the mice were anesthetized with ether. An incision was made in each animal to access the kidney under the capsule. A wedge shaped cavity was formed by excision of the renal cortex of each recipient kidney and a mass of tumor tissue of approximately 0.1×0.1×0.1 cm was placed under the renal capsule. A suture was then employed to secure the implant in place.

The five mice of this example were still alive six months later. Approximately one month following implantation of the tissue, the mice were surgically opened and the implanted tumors were observed. In each case, the tumor was found to have taken, i.e. the implanted neoplastic tissue had invaded adjacent tissue. Histological analysis was performed on the tissue implants at this time. Such analysis comprised removing tissue samples from each animal and comparing the samples with a tissue sample from the tissue donor.

Preparation of the tissue samples for histological analysis was carried out by (1) fixing the sample in formalin; (2) embedding the fixed sample in paraffin; (3) preparing 5-micron sections of the fixed, embedded sample; (4) staining the sections with hematoxylin and eosin; and (5) microscopically observing the tissue structure in each section.

Histological analysis revealed that the tissue in the recipient animals preserved its architecture and tissue type and mimicked progression of the disease in the human donor.

EXAMPLE II

In this example, specimens of human tissue excised from the stomach and pathologically diagnosed as gastric carcinoma were prepared to size by the teasing procedure described earlier.

Five athymic nude mice age four (4) to six (6) weeks were selected as the animal recipients for the implants. In preparation for surgery, the mice were anesthetized with ether.

Each anesthetized mouse was opened to provide access to the stomach. An incision was made in the stomach wall using a number 11 scalpel taking care not to penetrate the mucosal layer. A pocket was formed large enough to receive five tumor masses of about 0.1×0.1×0.1 cm each. A tumor piece of approximately this size was selected and inserted into the pocket and the incision was closed using a 7-0 suture.

The five mice of this example have survived for about three (3) to four (4) months and otherwise appear healthy. Subsequent surgical opening of the stomach of these mice has verified that the tumors have taken.

EXAMPLE III

In this example, specimens of human tissue removed from a human colon and pathologically diagnosed as colon carcinoma were prepared to size by the teasing procedure described earlier.

Five athymic nude mice, age four (4) to six (6) weeks were selected as the animal recipients for the implants. In preparation for surgery, the mice were anesthetized with ether. Each anesthetized mouse was opened to provide access to the colon. A pocket or cavity was surgically formed in the seromuscular layer with care exercised not to enter the lumen. Five to ten tumor masses of approximately 0.1×0.1×0.1 cm each were inserted into the pocket which was then closed with a suture.

Four of the five mice which underwent this implant surgery have survived for three to four months and appear to be in good health. Approximately one month following tissue implantation, the mice were surgically opened and the tumors were observed to have taken.

EXAMPLE IV

This example relates to the use of a vascularizing matrix to induce vascularization and take of orthotopically transplanted human tumor tissue.

A surgical tissue specimen, removed from a human colon and pathologically diagnosed as colon carcinoma, was washed with colon-wash medium. Necrotic tissue was removed and the tumor was then cut into small pieces (about 1-mm^3). Colon-wash medium, used to remove infectious intestinal material, was formulated by combining 500 ml of

Minimum Essential Medium with Earle's salts (MEM Earle's) with 70 ml fetal bovine serum, 75.2 mg Penicillin G sodium salt, 125 mg Streptomycin, 10 ml Fungizone antibiotic (250 ug amphotericin B and 205 ug sodium deoxycholate/ml in deionized distilled water), 5 mg Tetracycline, 50 mg Amikacin, 75 mg chloramphenicol and 50 mg Gentamycin.

GelfoamR brand of absorbable, sterile gelatin sponge (obtained from The Upjohn Co., Kalamazoo, Mich.) was hydrated with MEM Earle's. The hydrated sponge was cut into approximately 0.3–0.5×0.3–0.5×0.3–0.5 cm pieces which were transplanted onto the cecum of nude mice by means of a simple surgical suture on top of the cecum serosa. After 20 days, the sponges became well vascularized.

The transplanted vascularized sponges were cut in the center to make a pocket and about 10–15 of the previously prepared 1-mm^3 tumor pieces were implanted into each pocket which was closed by means of a surgical suture. The tumor grew locally and regional as well as liver metastases occurred.

EXAMPLE V

This example relates to the use of an internal skin flap to induce vascularization and take of orthotopically implanted human tumor tissue.

The tumor tissue used is identical to tissue used in Example IV and was prepared for implantation according to the procedure described in Example IV.

Skin flaps were constructed in the lower abdomen of nude mice by making incisions along three sides of a rectangular area (a U-shaped incision). The flap was lifted up and the abdominal wall was opened along the linea alba. The cecum was exteriorized from the abdominal cavity and tumor pieces (about 1-mm^3) were placed between the cecum serosa and the skin flap. Surgical sutures were applied along the two opposing edges of the flap to fix the flap on the cecum. The cecum, together with the skin flap was put back into the abdominal cavity. Peritoneum and rectus muscles were closed with sutures followed by reattachment of the skin flap with sutures. As a last step, surgical adhesive was applied to ensure good closure of the abdominal wall. The tumor grew at the implanted site and formed abdominal metastases.

EXAMPLE VI

This example relates to the use of a shish-kabob tissue configuration to effect orthotopic transplantation of human tumor tissue.

A surgical tissue specimen, removed from a human colon and pathologically diagnosed as colon carcinoma, was well washed with colon-wash medium. Necrotic tissue was removed and the tumor was then cut into small 1-mm^3 pieces. Eight of the 1-mm^3 pieces were assembled in a shish-kabob configuration by stringing the pieces together on a piece of surgical suture.

The shish-kabob tissue configuration was transplanted in nude mice by placement of the configuration on the mouse colon approximately 0.5 to 1 cm away from (i.e., up-stream of) the animals cecum. The configuration is held in place by securing the terminal ends of the suture material to the organ of the animal.

EXAMPLE VII

As shown below, local growth, regional metastasis, and in some instances, distant organ metastasis has been achieved using the three novel methods of orthotopic transplantation described in the present invention.

TABLE 1

Orthotopic Transplantation of Human Colon Carcinoma to the Colon of Nude Mice

Method	Number of Animals	Local Growth	Regional Metastasis	Distant Metastasis
Matrix	3	56–62 days	56–62 days	160 days
Skin Flap	3	48–62 days	48–62 days	
Shish-kabob	4	62–138 days	62–138 days	

EXAMPLE VIII

This example relates to the use of surgical adhesive to glue a piece of tumor tissue onto the top of the urinary bladder of the nude mouse.

A piece of tumor tissue specimen about 2-mm^3 size was prepared for tumor transplantation. The nude mouse was operated on under surgical anesthesia with the full exposure of the urinary bladder. A small amount of surgical adhesive (2-cyanoacrylic acid ester) was applied on the top of the urinary bladder and the previously prepared tumor piece was then glued onto the top of the urinary bladder. The abdomen was closed with surgical sutures.

Using the transplantation method described above, we transplanted the ras-transfected human bladder RT-10 carcinoma cell line xenograft. As a result, we achieved unexpected extensive growth and metastases, including invasion of the whole thickness of the urinary bladder wall, lymph node metastases, and multi-organ metastases in the liver, pancreas, spleen, ovary, kidney, ureter and lung. This transplantation result of RT-10 is in striking contrast to the result obtained when RT-10 was injected transurethrally as disaggregated cells where only local invasion and no distant metastasis were observed. (Theodorescu et al., Proc. Natl. Acad. Sci(1990), Vol 87, 9047–9051.)

EXAMPLE IX

In this example, a human tongue cancer specimen, which was prepared by being cut into 1-mm^3 pieces, was transplanted orthotopically to the floor of the mouth of a nude mouse.

An incision was made along the midline on the upper neck of the mouse. After blunt dissection of the muscles of the floor of the mouth, five pieces of prepared tumor tissue were implanted in between the muscles deep in the floor of the mouth. Surgical sutures were applied to close the dissected muscles and skin layer.

Extremely invasive growth was observed which involved the whole jaw as well as deep in the nasopharynx. This is distinctly different from growth observed when tumor pieces were implanted subcutaneously in the neck area. The subcutaneously grown tumor was completely encapsulated and exhibited no invasion of the adjacent tissue.

EXAMPLE X

In this example, a human pancreatic tumor specimen was prepared by being cut into 1-mm^3 pieces, and tumor pieces were transplanted onto the nude mouse pancreas.

A midline incision was made on the upper abdomen of the nude mouse muscle layers and the peritoneum were opened along the linea alba. Ten (10) pieces of previously prepared

tumor pieces were assembled in a shish-kebab configuration by stringing the pieces together on a piece of surgical suture. The configuration was secured on the pancreas. The abdomen was then closed with one layer surgical suture.

Two different kinds of human pancreatic cell line xenografts were transplanted as described above. Invasive growth was observed in both cases, including the invasion of the duodenum and spleen.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

What is claimed is:

1. A nude mouse model for human neoplastic disease, wherein said mouse is characterized by:

having histologically intact human neoplastic tissue of at least 1 mm^3 in size transplanted onto an organ of said mouse which corresponds to the human organ from which said tissue is originally obtained; and

having sufficient immuno-deficiency to allow said transplanted neoplastic tissue to grow and mimic the progression of the neoplastic disease in the human donor.

2. A nude mouse model according to claim **1** wherein said neoplastic tissue is selected from breast tissue, ovarian tissue or pleural tissue.

3. A nude mouse model according to claim **2** wherein said neoplastic tissue is obtained from human breast tissue.

4. A nude mouse model according to claim **3** wherein said human neoplastic breast tissue is implanted in the mammary fat pad of the mouse.

5. A nude mouse model according to claim **2** wherein said neoplastic tissue is obtained from human ovarian tissue.

6. A nude mouse model according to claim **5** wherein said human neoplastic ovarian tissue is implanted in the ovarian capsule of the mouse.

7. A nude mouse model according to claim **5** wherein said human neoplastic ovarian tissue is transplanted by securing to the surface of the mouse ovary at least two pieces of neoplastic tissue in close proximity to each other.

8. A nude mouse model according to claim **2** wherein said neoplastic tissue is obtained from human pleural tissue.

9. A nude mouse model according to claim **8** wherein said neoplastic tissue is implanted in the parietal pleura of the mouse.

10. A nude mouse model according to claim **9** wherein said neoplastic tissue is implanted in the visceral pleura of the mouse.

11. A method of generating a nude mouse model for human neoplastic disease, said method comprising:

transplanting histologically intact human neoplastic tissue of at least 1 mm^3 in size onto an organ of a nude mouse which corresponds to the human organ from which said tissue is originally obtained; and

allowing said transplanted tissue to grow and mimic progression of the neoplastic disease in the human donor.

12. A method of generating a nude mouse according to claim **11** wherein said human neoplastic tissue is selected from breast tissue, ovarian tissue or pleural tissue.

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CERTIFICATE OF SERVICE

I hereby certify that, on October 9, 2011, the foregoing document entitled **First Amended Complaint**, was filed via the Case Management/Electronic Case Filing (CM/ECF) system, with service to be made on all parties deemed to have consented to electronic service via the automated generation and e-mailing of a Notice of Electronic Filing (NEF) by the CM/ECF system.

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